THE RELATION OF DIET COMPOSITION AND VITAMIN C TO VITAMIN A DEFICIENCY[1]

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ONE FIGURE

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Previous experiments conducted by the authors on the onset and development of the vitamin A deficiency syndrome in the rat resulted in 2 major observations.

The earliest observed effect of vitamin A deficiency in young growing animals is to decrease the efficiency of utilization of the food consumed, this being defined by the ratio "grams body weight gained per calorie ingested" (Mayer and Krehl, '48a).

Some of the earliest symptoms of vitamin A deficiency seen in growing rats are part of a syndrome that responds favorably to the administration of ascorbic acid (Mayer and Krehl, '48b), despite the fact that under normal conditions the rat can synthesize adequate amounts of this vitamin. This response to ascorbic acid, together with the fact that the symptoms observed such as hemorrhage of the lachrymal glands, bleeding of the gums, and swelling of the joints with occasional paralysis of the limbs are similar to those seen in the scorbutic guinea pig, supports the view that these symptoms

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exhibited by our vitamin A deficient rats are indeed scorbutic in nature.

An artificial, so-called "synthetic" diet was used in both studies, with all known vitamins (except vitamins A and C) added to the diet as crystalline supplements. The experiments reported in this paper constitute an extension of these early observations. The first part of this report deals with the determination of the influence of the nature and proportion of various components of the diet on the rate of growth, the survival time and the efficiency of food utilization. It was reasoned that if any significant protection is afforded by a given foodstuff, or any injurious effect is obtained by increasing the concentration of another foodstuff, these phenomena might be regarded as indicating some relation of that foodstuff to the normal metabolic role of vitamin A.

The second part of this report deals with further study of the interrelationship of vitamins A and C. An attempt at integration of all the results is presented in the discussion.

VITAMIN A DEFICIENCY AND DIET COMPOSITION

Method

It has been shown (Mayer and Krehl, '48a) that in the female rat the decrease in efficiency of food utilization for growth as the course of vitamin A deficiency progresses, deviates less markedly from that of the controls and is more delayed in its appearance than is the case with the male. Because of this and other known effects of sex on the course of vitamin A deficiency in the rat, the first study of the influence of diet composition on vitamin A deficiency was restricted to 1 sex, and females were chosen arbitrarily. (For purposes of the growth assay for vitamin A, however, the male rat is considered preferable to the female.) Weanling female rats of the Sprague-Dawley strain were used. To insure a low vitamin A reserve in the experimental animals, the mothers received a diet low in vitamin A during the latter half of the nursing period. The weanlings were placed on experiment

when 24 days old. Seventy-two animals were used, 12 on each diet, with 8 deficient and 4 control animals in each one of the 6 groups. Each of the diets shown in table 1 represents a group.

The animals were kept in individual screen-bottomed cages at constant temperature and humidity. They were weighed every 4 days. All diets and water were fed *ad libitum*. The experiment was continued until all deficient animals had died.

TABLE 1

Composition of diets used.

INGREDIENTS OF BASAL MIXTURE [1]	DIET					
	A_1	A_2	A_3	A_4	A_5	A_6
	%	%	%	%	%	%
Casein [2]	25(25) [3]	10(10)	60(60)	39.4(25)	39.4(25)	25(25)
Corn oil	5(11)	5(11)	5(11)	7.9(11)	53.9(75)	5(11)
Sucrose	65.7(64)	80.7(79)	30.7(29)	.....	.....	.....
Dextrin	.....	.....	.....	.....	.....	65.7(64)
Lard	.....	.....	.....	46(64)	.....	.....
Salt mixture	4	4	4	6.3	6.3	4
Cystine	0.2	0.2	0.2	0.25	0.25	0.2
Choline	0.1	0.1	0.1	0.15	0.15	0.1

[1] Each of these basal mixtures was supplemented with the following vitamins in mg/kg (or mg/4,000 Calories for the high fat diets A_4 and A_5): α-tocopherol 100; 2-methyl-1,4-naphthoquinone 5; D_3 (viosterol) dissolved in corn oil 0.1; thiamine 5; pyridoxine 5; riboflavin 5; nicotinic acid 50; pantothenic acid 50; biotin 0.5; folic acid 0.5; inositol 100; para-aminobenzoic acid 100. Control rats received orally 1,200 I.U. vitamin A every 8 days.

[2] Vitamin-free "Labco."

[3] Figures in parentheses indicate the caloric distribution in per cent.

All diets used were of the "synthetic" type, being made up with purified ingredients and supplements of crystalline vitamins. The following major ingredients were used: Sucrose, dextrin, vitamin-free casein, refined corn oil, purified lard, and mineral salts mixture (Phillips and Hart, '35, modified by the addition of 0.02% $CoCl_2$). Detailed information on the exact composition of the diets used including vitamin supplements is given in table 1.

Diets A_1, A_2 and A_3 differed by the isocaloric replacement of sucrose with casein. A_6 was derived from A_1 by the replacement gram for gram of sucrose by dextrin to study the possible role of intestinal synthesis on vitamin A or its precursors. In diets A_4 and A_5 sucrose was replaced isocalorically by lard and corn oil, respectively, selected as examples of saturated and unsaturated fats.

Results

Data on the effect of diet composition on the survival time and the maximum weight attained by the deficient animals,

TABLE 2

Survival time and maximum body weight as affected by the composition of the diet.

RATION USED	VITAMIN A ABSENT		CONTROL
	Average survival time	Average maximum body weight	Body weight after 40 days
	days	*gm*	*gm*
A_2 (casein 10%, sucrose 79%)[1]	47 ± 4[2]	92 ± 9	166 ± 19
A_1 (casein 25%, sucrose 64%)	39 ± 3	109 ± 6	180 ± 19
A_3 (casein 60%, sucrose 29%)	30 ± 4	107 ± 6	158 ± 3
A_4 (casein 25%, lard 64%)	52 ± 9	145 ± 8	209 ± 18
A_5 (casein 25%, corn oil 75%)	45 ± 7	97 ± 10	182 ± 12
A_6 (casein 25%, dextrin 64%)	38 ± 2	119 ± 13	193 ± 18

[1] These percentages refer to distribution of calories.

[2] Average and standard deviation.

and, for reference, the weights of the control groups after 40 days on the experiment are presented in table 2.

Consideration of groups A_2 (10% casein), A_1 (25% casein), and A_3 (60% casein) makes it obvious that increasing the quantity of dietary protein decreases the survival time, even though the average maximum weights attained by the different groups are probably not significantly different. The use of dextrin instead of sucrose (group A_6) resulted in no benefit. The best results with respect to survival time, delay in onset of visible symptoms of vitamin A deficiency, and

average maximum weight attained by the deficient animals were obtained by the diet in which there was an isocaloric replacement of sucrose with lard (group A_4). The similar use of corn oil (group A_5) also resulted in some prolongation of survival time, although the average maximum growth of the deficient animals was very significantly inferior to that of the group fed the lard diet (A_4). This was true, although there probably was no significant difference between the weights of the control rats on the 2 diets.

It will be noted that all of these diets supported good growth in the control animals. Kidney function tests (creatinine and

TABLE 3

Effect of vitamin A deficiency on the efficiency of food utilization.

RATION USED	GRAM GAIN IN BODY WEIGHT			
	Per gm of protein eaten		Per calorie eaten	
	Deficient	Control	Deficient	Control
A_2 (casein 10%, sucrose 79%)[1]	1.67	2.35	0.04	0.06
A_1 (casein 25%, sucrose 64%)	1.35	1.59	0.08	0.10
A_3 (casein 60%, sucrose 29%)	0.42	0.65	0.06	0.10
A_4 (casein 25%, lard 64%)	2.7	2.8	0.10	0.10
A_5 (casein 25%, corn oil 75%)	2.0	2.5	0.08	0.10

[1] These percentages refer to distribution of calories.

para-aminohippuric acid clearances) were conducted on the control rats, all of which were found to be normal.

Data on the effect of vitamin A deficiency on the efficiency of utilization of the food ingested are given in table 3, expressed both in "grams of body weight gained per gram of protein intake" and in "grams of body weight gained per calorie ingested" after 20 days on the experimental diet. It will be noted, by comparing groups A_2, A_1 and A_3, that the efficiency of utilization of protein ingested by the control animals decreases when the proportion of casein in the diet increases. On the lowest level of dietary protein (group A_2), where the efficiency of utilization of dietary protein is high, the efficiency of food utilization is, however, lowest, appar-

ently because the animals increase their food consumption in order to ingest a sufficient amount of protein. If the protein level is sufficient to insure optimal growth (the animals on the low protein level are slow to start), the efficiency of food utilization in "grams body weight gained per calorie ingested" is the same on the various diets, in accordance with previous observations that specific dynamic action is not an important phenomenon in the growing animal.

A high protein intake makes more acute the decrease in efficiency of food utilization of the deficient animals compared to that of their controls. For example (table 3, diet A_3, casein 60%), the grams gained per calorie consumed were 0.06 for the deficient animals compared with 0.10 for their controls, a decrease of 40%. Similarly, for the medium level of protein (diet A_1) the drop was 20%. Unsaturated fat (corn oil, diet A_5) compares unfavorably with saturated fat (lard, diet A_4) in this respect. This point is considered in greater detail in the discussion.

The animals on the high protein, vitamin A deficient diet (A_3) died exhibiting very marked symptoms which are considered as being scorbutic in nature (see introduction for interpretation). On autopsy, evidence of extensive abdominal hemorrhage was observed.

One of the chief and uniformly characteristic autopsy findings in animals which died as a result of vitamin A deficiency (especially with added vitamin C) was a greatly bloated and distended gastrointestinal tract. For example, the volume of the distended stomach alone of 1 rat was 25 ml for a 100 gm animal. A clue to the cause of this distension was given in 1 case in which autopsy immediately after death revealed that the esophagus of the animal had completely separated from the stomach, which had become sealed off at the cardia. This extremely bloated condition of the intestinal tract can be seen in figure 1. This abnormal intestinal tract helps explain (1) why the vitamin A deficient animal stops eating completely several days prior to death, and (2) why autopsy findings reveal an almost complete depletion of depot fat

reserves. A histological examination of sections made from the esophagus and the stomach at the cardiac sphincter and of the duodenum revealed the following: (a) the lumen of the esophagus was completely occluded with cellular debris consisting mainly of keratinized epithelium — this occlusion

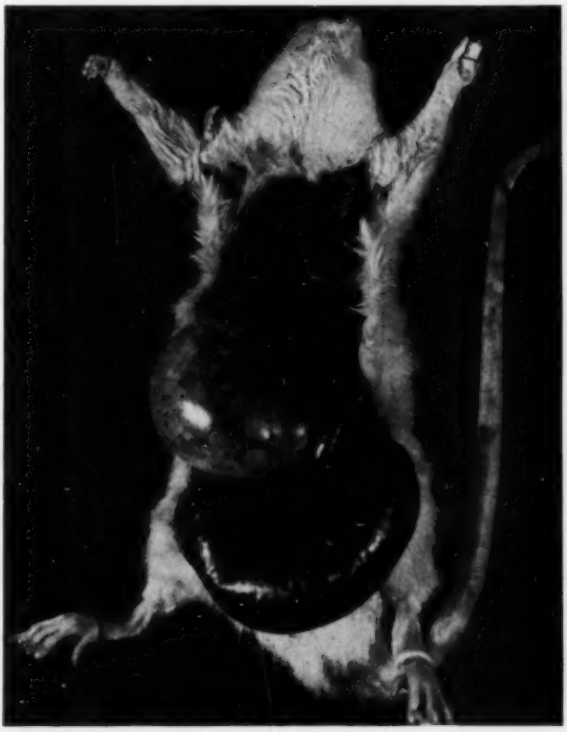

Fig. 1 Abdominal organs of a vitamin A deficient rat as seen at autopsy (dietary vitamin C added).

might have been the primary factor in causing the cleavage between the esophagus and the stomach; and (b) keratinization of the epithelium of the gastric and duodenal mucosa was evident. This was particularly marked in the duodenum, and in addition the height of the villi appeared to be less than

normal. A more complete pathological examination is being made and will be reported elsewhere.

In addition to these gastrointestinal symptoms which became evident only in the last stages of the deficiency prior to death, all the vitamin A deficient animals showed a deposition of dark red material around the nose and on the whiskers which was probably porphyrin in nature, as indicated by a characteristic fluorescence in ultra-violet light. As mentioned above, abdominal hemorrhage was often seen at autopsy but appeared less frequent and less severe in animals on the high fat rations.

Blood studies which were conducted on all deficient groups revealed a normal blood picture except in the high protein (A_3) group. In this group hemoconcentration (hematocrit, cell volume 60%) and a low white blood cell count (average 5,000 instead of 15,000 for the other groups) were observed.

In most female animals the decline in weight was not continuous, but showed first a decrease, then a period of temporary increase, and then a second fall in weight terminated by death. The period of temporary increase lasted about 4 to 6 days and corresponded in all cases with the onset of puberty, evidenced by opening of the vagina. This regain in weight varied between little more than a plateau in the weight curve to an increase of about 40 gm. If the weight of the animals was still increasing at the onset of puberty, seen particularly with the high lard diet (A_4), no such effect could, of course, be detected.

INTERRELATIONSHIP OF VITAMIN A DEFICIENCY AND VITAMIN C

Method

In the experiments dealing with the relationship between vitamins A and C, male rats of the Yale strain obtained from the Connecticut Agricultural Experiment Station were used first. They were weaned at 21 days and then placed on the experimental diet. The ration used was A_1 (table 1). When ascorbic acid was added, the proportion used was 100 mg per

100 gm of diet. The Roe-Kuether ('43) method was used for the determination of ascorbic acid.

Results

Twenty animals were used for blood and tissue determinations of ascorbic acid. They were given diet A_1 without vitamin C supplement. Control animals were given 1,200 I.U. of vitamin A every 10 days. Table 4 gives ascorbic acid values for the blood, adrenals and liver of these animals, both the deficient and the controls, after 37 days on the experimental regime. It will be seen that in all cases the values for blood and liver ascorbic acid of the vitamin A deficient animals are less than half of the normal. The adrenal ascorbic acid value is also very low.

TABLE 4

Ascorbic acid content of adrenals, liver and blood of vitamin A-deficient male rats and their controls.[1]

ANIMALS	ADRENALS	LIVER	WHOLE BLOOD
	mg/100 gm	*mg/100 gm*	*μg/ml*
Controls	355 ± 6(4)	34.5 ± 0.2(2)	16.1 ± 0.1(4)
Deficient in vitamin A	212 ± 35(11)	15.6 ± 1(3)	7 ± 1.5(11)

[1] Averages and standard deviations. Figures in parentheses indicate the number of animals studied.

It was also found, as might be expected in the few tests that it was possible to make, that the administration of vitamin C restored the adrenal, liver and blood ascorbic acid values to normal. Although the administration of vitamin A restored liver ascorbic acid to normal (28 mg per 100 gm), and had effected an increase in the adrenal ascorbic acid (average of 275 mg per 100 gm), the blood vitamin C level was still low (8 μg per ml).

In a second experiment, designed to test the effect of an addition of vitamin C to a vitamin A deficient diet on the development of vitamin A deficiency, 31 animals were used. The

animals were distributed in the following way (table 5): Four rats received vitamin A but no vitamin C, 10 received neither, 10 received both, and 7 received vitamin C but no vitamin A. The data in table 5 show that, although the control animals receiving vitamin A exhibited similar weight curves, the deficient animals behaved in a very different manner according to whether they were or were not provided with vitamin C

TABLE 5

Survival times and body weights of vitamin A-deficient rats and their controls with and without vitamin C in the diet.

ANIMALS	TIME ON EXPERIMENTAL DIET IN DAYS				
	20	30	35	40	80
Vitamin A-deficient rats					
Without vitamin C (10 animals)					
Body weight (gm)	159	137	175	...	...
Survival (%)	100	100	20	0	0
With vitamin C (7 animals)					
Body weight (gm)	148	212	229	245	302
Survival (%)	100	100	100	100	100
Control rats — received vitamin A					
Without vitamin C (4 animals)					
Body weight (gm)	225	303	343 [1]	373 [1]	...
Survival (%)	100	100	100	100	...
With vitamin C (10 animals)					
Body weight (gm)	214	304	328	354	505
Survival (%)	100	100	100	100	100

[1] The smallest of the original 4 control animals was sacrificed for analytical purposes. The survival and body weight data are therefore based on the remaining 3 larger animals.

in their diet. After 35 days on the experimental rations, all but 20% of the animals not receiving ascorbic acid had died; after 40 days all were dead. On the other hand, all of the vitamin A deficient animals which received vitamin C were still alive after 80 days. The maximum average body weight reached by the A-deficient animals not receiving ascorbic acid was 159 gm. The average weight of the vitamin A deficient

animals at 80 days was 302 gm, at which time the average weight of the controls was 505 gm.

That the effect of ascorbic acid in moderating the severe symptoms of vitamin A deficiency is not restricted to rats of Yale strain became evident when a group of 25 weanling male rats of the Sprague-Dawley strain (ordered specifically for purposes of vitamin A assay so as to have low vitamin A reserves) were placed on the vitamin A deficient diet (A_1); 80% of the animals have survived for 80 days and attained an average weight of 245 gm. No xerophthalmia or marked visible evidence of vitamin A deficiency is apparent in these surviving animals. Of 6 control animals on the same diet but receiving vitamin A, all have survived, and their average weight at 80 days was 370 gm.

DISCUSSION

It must be noted that although many studies have been made on the role of various diet constituents on vitamin A absorption and utilization (Sherman, '40; Russell et al., '41; Ahmad, '31; Dutcher et al., '34; Muelder and Kelly, '42), no systematic investigation has been made on the influence of diet composition on the vitamin A deficiency syndrome. Furthermore, no studies (to our knowledge) have been conducted on vitamin A deficiency in the rat with a purely artificial (synthetic) diet; most workers, for example, have used up to 10% yeast as a source of B vitamins. Although it is difficult at present to ascribe a great significance to the use of synthetic diets beyond their ease of reproducibility and chemical purity, we believe that the influences of the level of dietary protein, of dietary fat, and of the replacement of saturated by unsaturated fat are especially significant in studies on vitamin A deficiency.

In the interpretation of the effect of protein level, however, a complication arises from the existence of an interrelationship between vitamins A and C. Although decreases in blood ascorbic acid in young bovines and urinary excretion of this vitamin in the rat have been associated with vitamin A de-

ficiency (Boyer et al., '42), and similar relationships have been demonstrated for rat tissues (Sure et al., '39) and rat blood (Jonsson, Obel and Sjoberg, '42), the present and previous (Mayer and Krehl, '48b) experiments clearly point out that such findings are but mere reflections of the much more deep-seated pathological modifications that can be observed in the vitamin A deficient rat that does not receive added dietary or parenteral ascorbic acid.

As it has been demonstrated (Sealock and Silberstein, '39; Sealock, Perkinson and Basinski, '41, etc.) that ascorbic acid is needed for the metabolism of certain amino acids, phenylalanine and tyrosine in particular, it was theorized that perhaps the first underlying reason for the differences observed on the various protein levels is an increasing depletion of vitamin C caused by vitamin A deficiency, and that this is made more acute as the protein level is increased. This may explain why the deficient animals on the high protein diet in the first study in which vitamin C was not used died first and did so with very marked "scorbutic symptoms." It could also explain the fact that in the first experiment on the high protein diet the weight curves of the female control animals began to show a plateau when a weight of about 160 gm had been reached, after which there was a rapid gain in weight when ascorbic acid was added to the diet.

The experiment described in the first part of this paper on the influence of diet composition on vitamin A deficiency is now being repeated, but this time with vitamin C included in the diet. Preliminary results indicate that under these conditions the protein effect observed previously is in large measure cancelled. In fact, the effect of increasing the proportion of casein in the diet seems to be at least partly reversed, with the animals on the lowest level of protein showing the shortest survival time.

It seems logical to conclude, therefore, that the differences in survival time and efficiency of utilization on various levels of protein are not necessarily primary effects of vitamin A deficiency but secondary ones due to induced acute vitamin

C deficiency, which appears, as we have shown, many days, if not weeks, before acute symptoms of vitamin A deficiency are observed. On the other hand, the differences observed in the efficiency of food utilization between the animals fed a high saturated-fat diet (lard) and those given a high unsaturated-fat ration (corn oil) might well be a primary effect of vitamin A deficiency, inasmuch as certain authors (e.g., Monaghan and Schmidt, '32) have suggested that carotene and vitamin A may play a role in the oxidation of unsaturated fatty acids, and Sherman ('40) has indicated that there may be a metabolic interaction in vivo between vitamin A and linoleic acid.

The fact that an abnormally low white cell count was observed only in deficient rats fed a high level of protein suggests a possible interpretation of the controversial results obtained by various authors in their studies of the blood pictures of vitamin A deficiency in man. Hennessey ('32) and Sweet and K'ang ('35), for example, reported no effect of avitaminosis A on the white blood cell count, while Koessler, Maurer and Loughlin ('26) and Abbott and Ahmann ('38) found a decrease. We feel that possible differences in the composition of the diets used in these various studies might be responsible for this discrepancy.

The existence of a period of temporary recovery in body weight at the onset of puberty may be significant of the greater concentration in the blood of various hormones known to exist during that period. The possible influence of these hormones is now being investigated, together with the possibility that an increased rate of ascorbic acid synthesis may be involved.

The authors also feel it worthy of mention that a difference of efficiency of food utilization on the high saturated-fat diet was observed to develop more rapidly on a few animals placed in the cold (about 9°C.), which is known to cause an increased need for ascorbic acid (Therien and Dugal, '47).

It seems obvious that the existence of an interrelationship between vitamins A and C in the rat has an important bearing on any method of bioassay of vitamin A using this species.

SUMMARY

Rats were maintained on vitamin A deficient diets whose compositions were varied with respect to protein, carbohydrate and fat. The following observations were made:

1. In vitamin A deficient animals, increased levels of dietary protein resulted in decreased efficiency of protein utilization for growth, decreased efficiency of food utilization, decreased survival time, and a general increased severity of the symptoms of vitamin A deficiency.

2. The isocaloric replacement of sucrose by fats afforded an increased protection against development of the syndrome of vitamin A deficiency. This was particularly noticeable with saturated fat (lard).

3. One of the first symptoms of the vitamin A deficiency syndrome was a depletion of the animal's vitamin C reserves, as evidenced by symptoms resembling scurvy and curable by ascorbic acid, as well as by decreases in the ascorbic acid content of liver, blood and adrenals. Evidence is presented suggesting that at least part of the effects of an increased protein level on vitamin A deficiency is mediated through the concomitant decrease in vitamin C reserve.

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THE EFFECT OF ISOCALORIC SUBSTITUTION OF ALCOHOL FOR DIETARY CARBOHYDRATE UPON THE EXCRETION OF B VITAMINS IN MAN[1]

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The metabolism of ethyl alcohol by man supplies 7 calories per gram which can replace isodynamic quantities of carbohydrate and fat for energy (Atwater and Benedict, '02). There is evidence that ethyl alcohol is oxidized to acetaldehyde and that its further metabolism is coupled with pyruvic acid utilization (Leloir and Munoz, '38; Westerfeld, Stotz and Berg, '42, '43).

Clinical studies have associated "alcoholic" polyneuritis and pellagra with vitamin deficient diets (Shattuck, '28; Jolliffe, Colbert and Joffe, '36; Strauss, '35; Spies and DeWolf, '33). These reports did not show whether it was the lack of any specific vitamins required for the metabolism of the alcohol or the insufficiency of vitamins, protein and non-alcoholic calories which was responsible for the deficiency symptoms. Spies and DeWolf ('33) and Strauss ('35) were able to cure alcoholic pellagra and polyneuritis by feeding the subjects high caloric diets rich in good protein and B vitamin supplements, in addition to their daily whisky. In the report of

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Jolliffe, Colbert and Joffe ('36), it is seen that the caloric intake from non-alcoholic foods of most of the alcohol addicts with polyneuritis was grossly inadequate in contrast to that of the alcohol addicts without polyneuritis.

Lowry et al. ('42b) have shown a delayed development of polyneuropathy in rats on a thiamine deficient diet when alcohol was substituted isocalorically for part of the food intake, or when alcohol was added to the diet of pair-fed animals in lieu of water. Isocaloric replacement of alcohol for fat or for carbohydrate in the thiamine deficient ration of pigeons showed a similar sparing action of alcohol by its delay of onset of opisthotonus and death (Westerfeld and Doisy, '45).

In an effort to obtain further evidence concerning the relationship of alcohol metabolism in man to the requirements for thiamine and the other B vitamins, the present study was undertaken. The urinary excretion of several of the B vitamins was measured during experimental periods in which the only dietary change was the isocaloric substitution of ethyl alcohol for part of the carbohydrate (cornstarch and sugar) of the diet, with no significant alteration in the other dietary components.

EXPERIMENTAL

The subjects were 2 well-nourished adult males in good health (S and B, ages 31 and 44, weighing 75 and 82 kg, respectively). The composition of the experimental carbohydrate diet is shown in table 1. The daily menus were alternated as shown and were calculated to provide an adequate diet as summarized in table 1. In the given diet, the cornstarch, sugar and milk were served as puddings with the meals. This cornstarch and sugar supplied 1000 cal. per day and did not contain any appreciable amounts of vitamins, fat or protein. For the alcohol diet, four-fifths of the cornstarch and sugar were isocalorically replaced by 115 gm (800 cal.) of pure ethyl alcohol (150 ml of 95%). Water was added to give a volume of 10 oz. (approximate composition of whisky) and this was suitably diluted with water for consumption at the following

TABLE 1

Composition of carbohydrate diet.[1]

MENUS FOR ALTERNATE DAYS			
Food	Amount	Food	Amount
	gm		gm
Breakfast			
Egg	50	Egg	50
Bacon	20	Bacon	20
Orange juice	120	Orange juice	120
Toast	50	Toast	50
Butter	10	Butter	10
Coffee	180	Coffee	180
Dinner			
Ground meat, cooked, wt.	100	Roast beef, cooked, wt.	100
Onions	50	Lettuce	30
Lettuce	30	Tomatoes	50
Asparagus, canned	50	Mayonnaise	14
Mayonnaise	14	Green peas, canned	100
Carrots, canned	100	Bread	50
Bread	50	Oleo	10
Oleo	10	Cornstarch	35
Cornstarch	35	Sugar	95
Sugar	95	Milk	240
Milk	240		
Supper			
Mor beef, canned[2]	100	Mor beef, canned	100
Corn, yellow, canned	100	Spinach, canned	100
Potatoes, cooked, wt.	150	Potatoes, cooked, wt.	150
Bread	50	Bread	50
Oleo	25	Oleo	25
Cornstarch	35	Cornstarch	35
Sugar	95	Sugar	95
Milk	240	Milk	240

[1] The above diet supplied the following calculated daily average food values: calories 3275; protein 88 gm; fat 139 gm; carbohydrate 417 gm; vitamin A 13,000 I.U.; ascorbic acid 100 mg; thiamine 1.3 mg; riboflavin 2.2 mg and niacin 15.5 mg.

[2] This was generously supplied by Dr. E. J. Czarnetzky of Wilson and Company, Inc., Chicago.

times: 2 oz. each before dinner and supper, 1 oz. at mid-afternoon, and 5 oz. during the evening.

During the experimental period, the carbohydrate diet was consumed for 7 days, the alcohol diet for the next 10 days, and the original carbohydrate diet for the last 6-day period. Beginning with the second day of the diet, 24-hour urines were collected (kept in the refrigerator with 15 ml of glacial acetic acid) and pooled in 48-hour periods for analysis of thiamine, riboflavin, nicotinic acid, N^1-methylnicotinamide, pantothenic acid, 4-pyridoxic acid, pteroylglutamic acid, tryptophane, creatinine and nitrogen.

Thiamine was measured by a modification of the thiochrome method using a sulfite blank (Mason and Williams, '42). N^1-methylnicotinamide and 4-pyridoxic acid (as the lactone) were also measured fluorometrically by modifications of the methods of Huff and Perlzweig ('43, '44).

In the microbiological methods used for determination of riboflavin, nicotinic acid, tryptophane, pantothenic acid and pteroylglutamic acid, 40 gm each of glucose and of sodium acetate were used per liter of medium. *Lactobacillus casei* was used (1) for riboflavin assay with this change in the medium of Snell and Strong ('39) and (2) for pteroylglutamic acid assay with a medium similar to that of Mitchell and Snell ('41). The medium of Krehl, Strong and Elvehjem ('43) was employed with *Lactobacillus arabinosus* as the test organism for assay of nicotinic acid and tryptophane. When used for tryptophane analysis, this component of the medium was replaced by 400 μg of nicotinic acid per liter. *Lactobacillus arabinosus* was also used for measurement of pantothenic acid (Skeggs and Wright, '44). All microbiological tests were titrated with 0.1 *N* NaOH (bromthymol blue as indicator) after incubation at 37°C. for about 65 hours.

Aliquots of the urines were digested with sulfuric acid and 30% hydrogen peroxide for determination of total nitrogen with the use of Nessler's reagent. Creatinine was measured by a simple colorimetric procedure using alkaline picrate re-

agent. A photoelectric colorimeter was used for these readings.

RESULTS

The urinary excretion of the substances mentioned above is shown in table 2 for the 2 subjects while on the carbohydrate and alcohol diets. The excretion of creatinine was relatively constant during the experimental period and attests to the completeness of sample collection. Subject S started the experiment at a higher level of nitrogen excretion than did subject B, but later the excretion of nitrogen by the 2 subjects was almost the same. The change from carbohydrate to alcohol diets did not significantly affect the nitrogen excretion of either subject. This is paralleled by the relatively constant tryptophane excretion. Daily weights of the subjects showed less than 1 pound variation from the initial weight during the experiment. No attempt was made to control fluid intakes, and minor variations in urinary volume occurred.

The excretion of thiamine by both subjects increased markedly after changing from the carbohydrate to the alcohol diet, and decreased correspondingly when the carbohydrate diet was resumed. This may be an indication of lower thiamine requirements when carbohydrate is replaced by alcohol in man. Other evidence for a thiamine sparing action of alcohol has been presented for rats (Lowry, '42b) and pigeons (Westerfeld and Doisy, '45). The urinary N^1-methylnicotinamide was also greatly increased on the alcohol diet in the present experiments, and decreased upon resumption of the carbohydrate diet. Small changes in the nicotinic acid excretion appear to follow the pattern of changes of N^1-methylnicotinamide excretion on these diets. These results may indicate similar changes in thiamine and nicotinic acid utilization in alcohol metabolism.

The riboflavin excretion of subject S was much higher than that of subject B at the beginning of the experiment. This might be correlated with the higher initial nitrogen excretion of this subject whose diet contained more milk prior to the experiment. Both subjects showed variations in riboflavin

TABLE 2

Daily urinary excretion of vitamins and other metabolites by 2 subjects on carbohydrate and alcohol diets.

URINARY EXCRETION	SUBJECT	CARBOHYDRATE DIET			ALCOHOL DIET					CARBOHYDRATE DIET		
		Days			Days					Days		
		2–3	4–5	6–7	8–9	10–11	12–13	14–15	16–17	18–19	20–21	22–23
Thiamine (μg)	B	93	66	66	104	111	111	147	190	98	91	80
	S	80	70	68	99	111	104	116	118	77	80	60
N^1-Me [1] (mg)	B	4.5	3.7	4.5	6.3	5.5	6.4	6.5	6.9	6.0	5.8	4.6
	S	6.5	5.6	5.0	5.8	7.3	7.5	9.0	10.2	8.5	8.8	4.7
Nicotinic acid (mg)	B	0.9	0.8	1.1	1.3	1.2	1.4	1.2	1.6	1.1	0.9	0.8
	S	1.0	0.8	1.0	1.4	1.3	1.2	1.1	1.6	0.9	0.9	0.8
Riboflavin (μg)	B	340	490	510	750	555	500	540	570	380	480	540
	S	1180	950	975	1090	700	510	470	550	380	540	600
4-Pyridoxic acid (mg)	B	1.4	29.6	28.5	18.8	3.5	4.1	4.0	8.0	4.3	35.3	29.3
	S	4.8	28.0	32.5	20.0	3.6	3.0	2.8	7.3	3.2	34.7	10.0
PGA [2] (μg)	B	2.7	3.0	2.5	3.2	3.0	2.7	3.2	3.1	2.5	2.5	2.9
	S	3.4	3.4	3.0	3.5	3.6	3.7	3.3	3.2	3.0	3.0	3.2
Pantothenic acid (mg)	B	3.1	3.2	2.8	3.4	2.5	2.5	2.6	2.8	2.2	2.5	2.7
	S	4.8	4.7	4.1	4.8	4.3	3.6	3.9	4.1	3.2	3.6	3.6
Tryptophane (mg)	B	26	24	23	23	21	24	23	24	22	23	23
	S	20	20	19	19	24	21	20	21	20	20	20
Creatinine (gm)	B	2.0	2.0	2.1	2.2	1.9	2.2	2.1	2.1	1.9	2.1	2.1
	S	2.2	2.2	2.3	2.3	2.5	2.3	2.3	2.3	2.1	2.2	2.2
Nitrogen (gm)	B	11.2	12.3	12.9	13.3	13.7	14.6	13.2	13.7	11.0	12.8	12.3
	S	15.9	15.1	14.8	16.3	12.0	14.2	13.6	14.5	13.2	13.4	13.2
Urine volume (ml)	B	1520	1450	1170	1370	1330	1390	1430	1530	970	1070	960
	S	1190	1150	1250	1270	1200	1500	1170	1510	1100	1030	1230

[1] N^1-methylnicotinamide.

[2] Pteroylglutamic acid.

excretion which are not readily interpretable in regard to requirement for alcohol and carbohydrate metabolism. The experimental periods may have been too short to reflect differences, if any, in riboflavin requirements on these diets.

The excretion of 4-pyridoxic acid is an indication of the metabolism of the pyridoxine complex (Huff and Perlzweig, '44; Johnson, Hamilton and Mitchell, '45) but the level of excretion has little meaning in human nutrition at the present time. Johnson et al. ('45) have reported an average daily excretion of 3.5 mg of 4-pyridoxic acid, and in the authors' laboratory a range of 1 to 5 mg per day is usually found for normal subjects. On the initial carbohydrate diet, the excretion of this substance by both subjects started in the normal range and increased to about 30 mg per day. This value decreased on the alcohol diet and approached the normal level. The final carbohydrate diet again resulted in this unexplainable excretion of high levels of the acid. Since these values were obtained by fluorometric analysis, it is possible that some unknown metabolite of the carbohydrate diet was interfering with the analysis. The sugar and cornstarch, which were consumed on the carbohydrate diet, contained insignificant amounts of vitamin B_6. The cornstarch [3] contained 0.07 μg of vitamin B_6 per gram as measured [4] by the method of Atkin et al. ('43). The 70 gm of cornstarch therefore contributed only about 5 μg of pyridoxine compounds to the diet. Another possible explanation for the high values is that a pyridoxine compound was synthesized in large amounts by intestinal bacteria in the presence of excessive cornstarch and sugar in the diet, but this was not reflected in the excretion of any of the other vitamins.

The excretions of both pteroylglutamic acid and pantothenic acid remained constant during the experimental period and were not affected by changes in the alcohol or carbohydrate contents of the diet.

[2] Pteroylglutamic acid.

[3] Powdered Buffalo cornstarch, Corn Products Sales Co., New York.

[4] This analysis was made through the courtesy of Dr. S. H. Rubin of Hoffmann-LaRoche, Inc.

[1] N'-methylnicotinamide.

DISCUSSION

Urinary levels of vitamins and other metabolites are resultants of many processes in the body. Therefore, the increased excretion of thiamine and N^1-methylnicotinamide which accompanied the isocaloric substitution of alcohol for carbohydrate in the present studies may have been caused by one or more factors. On the basis of our present knowledge of vitamin excretion, these results may be interpreted as suggesting a thiamine and nicotinic acid sparing action of alcohol. The increases in excretion do not appear to be attributable to the breakdown of tissue protein (Sarett, Klein and Perlzweig, '42) since the subjects maintained their weight and showed little change in the excretion of nitrogen and several of the other substances measured. It is possible that liver cell injury (Lowry et al., '42a) may have released enough thiamine and nicotinic acid to account for the extra vitamin excretion, with small nitrogen loss which would not be detectable in the present experiments. However, the excretion of the other vitamins, which should have been released at the same time, did not follow the patterns for thiamine and N^1-methylnicotinamide.

The thiamine sparing action of alcohol, when this substance is substituted for an equicaloric amount of a thiamine deficient diet for rats, has been shown by Lowry et al. ('42b) by a delayed development of polyneuropathy and death. Similar conclusions were reached by Westerfeld and Doisy ('45) who were able to delay the onset of opisthotonus in pigeons by substitution of alcohol for either fat or carbohydrate in a thiamine deficient ration.

It is difficult to understand how alcohol can exert a thiamine and nicotinic acid sparing action since its metabolism is thought to be linked with that of pyruvic acid, requiring both thiamine and nicotinic acid-containing enzymes (Leloir and Munoz, '38; Westerfeld, Stotz and Berg, '42, '43). It is possible that other pathways of alcohol metabolism may exist. However, even the assumption that alcohol requires no thiamine for its metabolism would not completely explain the following findings. The substitution of alcohol for fat (which

supposedly requires little or no thiamine for its metabolism) has a further thiamine sparing action in pigeons (Westerfeld and Doisy, '45), and the *addition* of alcohol to the thiamine deficient diet of pair-fed rats has a thiamine sparing action (Lowry et al., '42b). Westerfeld and Doisy ('45) suggested that the metabolism of carbohydrate and alcohol together may require less thiamine than does the metabolism of carbohydrate alone. This would be equivalent to a negative thiamine requirement of alcohol.

The indications from the above animal experiments and the present human studies may not be applicable to cases of excess alcohol intake with a low caloric intake of other foods.

SUMMARY

The isocaloric replacement of carbohydrate (sugar and cornstarch) by ethyl alcohol in an adequate diet resulted in an increased excretion of thiamine and N^1-methylnicotinamide in 2 human subjects. No significant changes were found in the excretion of creatinine, nitrogen, tryptophane, pantothenic acid and pteroylglutamic acid.

These data may suggest that the substitution of alcohol for carbohydrate has a thiamine and nicotinic acid sparing action.

The excretion of 4-pyridoxic acid on the carbohydrate diet was increased to unexplainably high levels.

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GROWTH AND REPRODUCTION OF RATS FED ARMY COMBAT RATIONS[1]

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INTRODUCTION

Extensive studies have been made of the composition of army combat rations. These analyses for carbohydrate, protein, fat and vitamins indicated that the daily intake was sufficient to supply the daily dietary requirements (QMC studies, '44). Very little work has been done on the feeding of these rations to experimental animals. The requirement for growth of an animal, such as the rat, would be higher for certain essential nutrients than that needed by man for maintenance. Nevertheless, animal feeding experiments might yield information which would be of value in improving the formulation of rations for man.

EXPERIMENTAL

The combat rations were those used by the U. S. Army and supplied by the Quartermaster Food and Container Institute for the Armed Forces. The C ration had been designed specifically for men in actual combat where no kitchen facilities

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

The subject matter of this paper has been undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

were available. It was packed in 6 small cans. Three cans, 1 for each meal, contained the meat components and offered any one of 10 different varieties, such as chicken and vegetables, ham and lima beans, pork and rice, and meat and spaghetti. The 3 other cans, again 1 for each meal, contained the bread components and included in toto biscuit, beverage powder, sugar, confectionery, pre-mixed cereal and jam.

The 10-in-1 ration was intended to serve troops in all areas where they were in advance of the field kitchen but sufficiently protected to permit small group feeding. It provided food for 10 men for 1 day. Each case of 10-in-1 ration contained 1 of 5 distinct menus, thus permitting each soldier 15 different consecutive meals. The 5 menus contained various components of canned meat items, biscuits, puddings, cereals, jams, vegetables, butter, sugar, milk, beverage powder, and confection (QMC studies).

The K ration was created to provide a ration light in weight, yet containing the essential nutrients. Each meal included approximately 4 ounces of either meat, meat and egg product or cheese spread, together with biscuits, confection, and beverage powder with sugar (QMC studies).

The components of the ration were ground in a meat grinder and thoroughly mixed in a Hobart mixer. Storage of all rations in the refrigerator reduced the possibility of rancidity. When the rations were supplemented with the B-complex, the following levels in milligrams per 100 gm of ration were used: thiamine 0.3, riboflavin 0.3, niacin 2, pyridoxine 0.2, pantothenic acid 2, folic acid 0.025, biotin 0.01, inositol 10, choline 100, and p-aminobenzoic acid 25. In cases where the vitamins were fed by dropper, the amount administered per day was equivalent to that included in 10 gm of ration.

In order to determine the ability of the ration to support reproduction and lactation in rats, the unsupplemented rations were fed to female weanling rats (Sprague-Dawley). Six rats were used in each group, and they received the ration and fresh water *ad libitum*. The animals were kept in separate cages until the tenth week of experiment, then each group

was placed in a large cage. At the end of the thirteenth week, females were mated with males from our stock colony.

The methods used in handling rats on reproduction experiments have been described previously (Sporn et al., '47). The females were remated after a 3-week rest period. The results obtained were presented as the total of both matings (table 1).

TABLE 1

Growth and reproduction studies on rats fed army rations.

	RATION			
	K	C	10-in-1	K + ½ gm raw liver/day
Female rats				
% conception	94	100	100	83
% stillborn	20	20	20	17
% survival of young	8	49	29	65
Male rats				
Average weekly wt. gain (gm) at end of:				
Fourth week	18	21	19	..
Seventh week	22	26	22	..

The experiments were repeated with double the number of animals in each group, and approximately the same results were obtained. Since the preliminary results showed K ration to be the poorest in supporting survival of young, raw liver was added to the diet of rats receiving this ration. It had a very pronounced effect in increasing the survival of young born to females fed this diet (table 1).

The inability of rats receiving the combat rations to support the survival of young indicated that the rations might be inadequate in known or unknown nutritional factors. Growth studies were undertaken to examine this possibility, since they constitute a faster method of analysis.

Male weanling rats (Sprague-Dawley) weighing between 35 and 40 gm were used in all growth experiments. They were housed in individual cages and were fed the ration and fresh

water *ad libitum*. Six rats were used in each group. Each series was repeated at least once.

Feeding the K, C, and 10-in-1 rations, respectively, to groups of male rats indicated that normal growth was not obtained (table 1). It is interesting to note that the average gain per week was increased when the rats were kept on experiment for a longer period.

TABLE 2

Effect of supplements added to K ration fed to rats.

GROUP [1]	B VITAMINS	A AND D	CASEIN	SALTS IV	LIVER	AVERAGE GAIN/WEEK AT END OF 4TH WEEK
						gm/week
1		+				19
2			+			20
3				+		19½
4					½ gm raw liver/day	24
5	Twice weekly by dropper					20
6	In ration	+				23
7	In ration	+			3% W.L.S.[2]	24
8	In ration	+	+		3% W.L.S.	33
9	In ration	+	+	+	3% W.L.S.	36
10	Double level in ration					26
11	Double level in ration			+		25
12	Double level in ration		+			34
13	Double level in ration		+	+		36
14	Double level in ration		+		ethanol extract of liver	37
15	Double level in ration		+	+	ethanol extract of liver	36

[1] Five rats per group.

[2] Whole liver substance.

Since the K ration gave poor growth and was easily prepared for feeding, it was decided to work more intensively with this ration than with the others. Vitamins A and D, as oleum percomorphum diluted 1:4 with corn oil and fed at the rate of 2 drops per week, 5% casein, or 2% salts IV had no effect when added individually to the diet of rats on K ration (table 2, groups 1, 2 and 3). When the B-complex was fed by

dropper, no effect upon growth was noted (group 5). This addition, when mixed in the ration, brought about a growth response equivalent to feeding ½ gm of raw liver per day (groups 4 and 6). Supplementation with whole liver substance did not produce a response above that observed with the B-complex (group 7).

When K ration was supplemented with the water-soluble vitamins as well as casein,[2] good growth was obtained (group 12). The addition of salts IV produced a small but consistent weight increase when included with these supplements (groups 9 and 13). When an ethanol extract of liver (Jaffé and Elvehjem, '47) was added to this improved diet as a source of unknown factors, no greater gain could be observed (groups 14 and 15).

The marked improvement with casein suggested an amino acid deficiency. With K ration containing a double level of B vitamins as the base of reference, individual amino acids were fed at 0.3% of this diet. The addition of phenylalanine had no effect upon growth; lysine had an inhibitory effect, the rats averaging 5 gm a week less than those on the basal diet; methionine caused an increased growth of 4 gm a week per rat above that for the basal group. When all 3 amino acids were added to the diet, again no effect upon the growth rate was observed. The effects of lysine and methionine seem to counteract each other when fed simultaneously.

On the basis of the information gained from experimentation with the K ration, the other combat rations were supplemented with similar factors. The C ration was not improved with the addition of vitamins A and D (table 3). The B vitamins produced a slight increase in the growth rate, but casein was necessary before fair growth could be obtained with this diet.

In the case of 10-in-1 ration, the water-soluble vitamins produced a small increase in the rate of gain. Even with both vitamins and casein, the growth rate was still suboptimum (table 3).

[2] Smaco brand.

Animals on most of the diets appeared normal at the end of the experimental period. The rats on unsupplemented K and 10-in-1 rations were an exception. Some of them showed a characteristic loss of hair. If they were allowed to continue on the particular diet for longer periods, there was a spontaneous recovery from this condition. Autopsy did not reveal any gross irregularities among any of the animals.

TABLE 3

Effect of supplementation of army rations upon rat growth.

RATION [1]	AVERAGE GAIN/WEEK AT END OF 4TH WEEK
	gm/week
C ration alone	21
C ration plus vitamins A and D	21
C ration plus vitamins A and D plus B vitamins	24
C ration plus vitamins A and D plus B vitamins plus 5% casein	33
10-in-1-ration alone	18
10-in-1 ration plus vitamins A and D	18½
10-in-1 ration plus vitamins A and D plus B vitamins	22
10-in 1 ration plus vitamins A and D plus B vitamins plus 5% casein	27

[1] Five rats per group.

The effect of storage upon the nutritional quality of the rations was also investigated. The K, C and 10-in-1 rations which had been returned from overseas were fed to groups of rats. In all cases, results indicated that these rations supported a growth rate comparable to that observed with fresher rations.

DISCUSSION

The results obtained from the *ad libitum* feeding of the combat rations to rats indicate that all of the rations were unable to support growth or survival of young in this species. The materials fed represented only a limited sampling since the items included in the rations were changed from time to time. Therefore, samples taken from other manufacturing sources and at different times might give results varying in

some degree from those reported in this instance. Furthermore, the rations were intended primarily for maintenance whereas growth and reproduction were the 2 criteria used in these experiments for evaluating the several rations. The growing animal would have a higher requirement for certain essential nutrients. The experimental data showed that when the animals were fed the rations for some time, a greater gain per week was observed at later periods. This indicates that the rations were better suited to support growth of the older animals, which have a lower requirement for certain nutrients.

Work which appeared recently tends to emphasize the difference in requirements between species. Cox et al. ('47) studied the effect of adding methionine to an enzyme casein hydrolysate fed to rats, dogs and man, and found that the requirement for methionine in man is lower than that for the dog. This was in confirmation of work published by Johnson et al. ('47).

It is seen from the results that none of the combat rations supported good growth or survival of young in the rat. From the studies on growth, it is observed that with the addition of a mixture of the B vitamins and casein, the K ration was improved so that normal growth was obtained.

Similarly, growth of rats upon C ration can be improved by the addition of the B complex and casein. Here again, the effect of casein is most striking. It is possible that the K and C rations are deficient in similar amino acids. Then again, casein may also be supplying unknown factors. Work from this laboratory (unpublished data) indicates that the casein used in this study contains an unidentified factor for the rat.

The 10-in-1 ration differs from the other rations tested. Though a combination of the B complex and casein exerts a beneficial effect, normal growth in the rat was not obtained.

When raw liver was fed as a supplement to the K ration, the results indicated improvement in growth and lactation. This may be attributed to added vitamins and proteins in the liver. It is interesting to note that a rather high level of vitamins in the diet is necessary to produce the maximum

response. It is a fairly well-established fact that best results are obtained when the vitamins are fed in the diet, and not as supplements in concentrate form at certain periods.

Supplementation with vitamins A and D had no effect upon the growth of rats fed any of the combat rations. Salts IV, when fed in addition to the B vitamins and casein, produced a slight stimulation of growth in rats on K ration. This increased growth rate was not large enough to be significant. The liver extract (Jaffé and Elvehjem, '47) was similarly inactive in increasing the rate of growth of the rats.

The phenomenon of loss of hair has been observed by other workers. Spitzer and Phillips ('46) observed a similar loss of hair in rats fed a corn-soybean ration, which was overcome by feeding inositol or biotin. It is believed that this condition is brought about by factors which influence the intestinal flora. The flora is prevented from supplying adequate amounts of some factor, probably biotin. In the case of rats receiving army rations, this condition is overcome without adding the necessary factors to the diet.

Work by Dunn et al. ('47) indicated that the K ration was low in phenylalanine, lysine and methionine, if compared to levels established by Rose as minimum for the rat. When these amino acids were fed as the probable source of activity in casein, only the methionine gave a positive response. Lysine produced an inhibition of growth. They nullified the effect of each other when fed as a mixture. This establishes only methionine as being necessary for increased growth of the rat on K ration. A more intensive study of amino acids as supplements to the K ration is contemplated.

SUMMARY

The army combat rations K, C and 10-in-1 were fed *ad libitum* to young growing rats. The materials fed represent only a limited sampling since the items included in the rations have been changed from time to time. Therefore, the samples

taken from other manufacturing sources and at different times might give results varying in some degree from those reported in this instance. Furthermore, growth and reproduction over extended periods of time were the 2 criteria used in these experiments for evaluating the rations. Actually these rations were consumed for the most part by mature men in the field during relatively short intervals of time.

When the K, C and 10-in-1 rations were fed to female rats, it was found that the survival rate of the young born to the females was quite low. The addition of liver to the K ration improved the percentage of survival of young born to females on this diet.

The growth of male rats on these diets was suboptimum. The addition of the B vitamins and casein to the rations substantially improved growth in all cases. Normal growth was obtained when K and C rations were supplemented with casein and vitamins. Results indicate that part of the response to casein with the K ration is due to the methionine content.

ACKNOWLEDGMENTS

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STUDIES WITH MONKEYS FED ARMY COMBAT RATIONS[1]

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TWO FIGURES

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INTRODUCTION

Extensive studies have been made of the chemical composition of army combat rations, with special emphasis on the vitamin content, as a guide to their formulation and improved methods of storage. Practically no work has been done on the feeding of these rations to experimental animals. In order to guide ration development, it is important to know whether the combination of foodstuffs theoretically capable of providing an adequate diet is actually complete when used for extended periods of time.

Recent work indicates very clearly that the requirements for different vitamins, and probably amino acids, differ considerably depending on the type of ration fed. Thus, the kind and level of carbohydrate and fat may substantially alter the vitamin requirements. Such changes in requirements can only be observed through controlled feeding experiments. This

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paper presents results which have been obtained on feeding army combat rations to rhesus monkeys.

There has been little work to correlate the nutritional requirements of man and monkey. Though the monkey is similar to man anatomically, its nutritional requirements may differ in several respects. Nevertheless, evidence may be obtained which will be of value in planning improvements in the ration.

Studies during the past 6 years have yielded much information regarding the use of monkeys in nutritional experiments. Waisman, Rasmussen, Elvehjem and Clark ('43) described a purified diet which is capable of maintaining monkeys in excellent health for extended periods of time. With the use of this diet, it has been possible to study uncomplicated vitamin deficiencies. Studies on thiamine (Waisman and McCall, '44), riboflavin (Waisman, '44), pyridoxine and pantothenic acid (McCall et al., '46), biotin (Waisman et al., '45), folic acid (Cooperman et al., '46a) and ascorbic acid (Shaw et al., '45) deficiencies have already been reported. Recent work has described an unknown factor which is necessary for continued growth and a normal blood picture in monkeys (Ruegamer et al., '47).

EXPERIMENTAL

The housing, care and handling of the monkeys have been described previously (Waisman et al., '43). The army combat rations K, C and 10-in-1 were prepared for feeding at 2-week intervals. The components were ground in a meat grinder, thoroughly mixed in a Hobart mixer and stored in a cold room. The monkeys were fed twice daily, and each feeding consisted of the contents of a full 1-pound butter crock. There was food in the cups at all times.

The army combat rations have been described in a previous publication (Sporn and Elvehjem, '48). The vitamin supplement was fed at the beginning of each day, and the monkeys usually drank this at once. No water was given to them until they had consumed this supplement. When milk was fed, it

was added after the vitamins were finished and before water was given to the animals.

The "mixture of water-soluble vitamins" referred to in the experiments consisted of the following (in milligrams) fed each day: thiamine hydrochloride 1, riboflavin 1, pyridoxine hydrochloride 1, calcium pantothenate 3, nicotinic acid 5, choline chloride 25, biotin 0.02, folic acid 0.1, p-aminobenzoic acid 50, i-inositol 50, and ascorbic acid 25. When individual vitamins were fed, they were administered at the levels noted.

Hemoglobin and differential leucocyte determinations were performed throughout the study. Samples were taken from blood drawn from the marginal vein of the ear. Hemoglobin determinations were made by the method of Evelyn ('36).

RESULTS

C ration

The C ration supported good growth in monkeys for about a month. At the end of this period, there occurred a decline in weight which lasted about 2 months and became more rapid at the end of the second month. All indications were that the monkeys would die without supplementation. With the addition of a mixture of the water-soluble vitamins, there was an immediate response in weight. When the vitamins were continued as the only addition to the ration, fairly good growth of monkeys was obtained for at least 1 year. The addition of 100 ml of raw milk per day did not improve the rate of growth observed after the supplementation with the vitamins. Typical growth curves of monkeys on C ration are shown in figure 1. Both monkeys appeared normal at the end of the experimental period.

The hemoglobin concentration of the blood was below normal after the monkeys had been on the C ration for 6 weeks. It took 1 to 2 weeks for the hemoglobin concentration to respond to the addition of vitamins to the diet. A maximum hemoglobin concentration was reached, in all cases, within 1 month after supplementation. The hemoglobin remained in

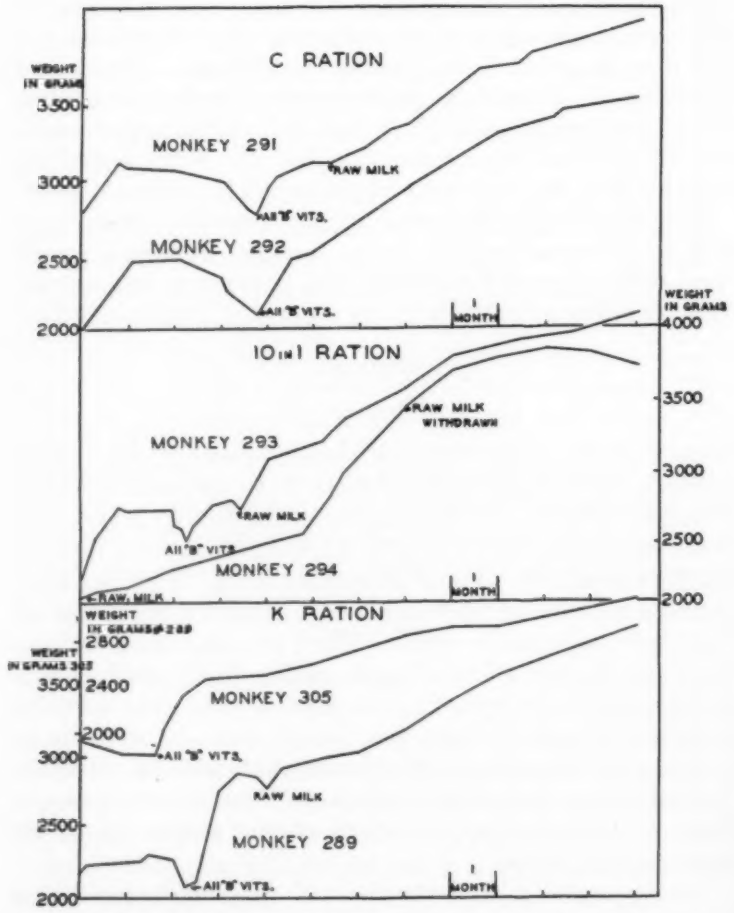

Fig. 1 Typical growth curves for monkeys fed the C, 10-in-1 and K rations. The additions of a mixture of the water-soluble vitamins, as well as 100 ml of raw milk per day, are indicated. Once added to the diet, the supplements were fed daily thereafter.

the normal range for the duration of the experiment, even though it fell slightly about 2 months after supplementation (table 1). The differential leucocyte count was normal at all times.

TABLE 1

Typical hemoglobin concentrations in monkeys on the C ration.[1]

	MONKEY NO. 291	MONKEY NO. 292
	gm %	*gm %*
Start of experiment	14.5	13.5
At the end of 6 weeks	12.5	13.5
Two weeks after vitamin supplementation was started	15.9	16.8
Two months after vitamin supplementation was started	14.2	14.7
After 1 year on experiment	14.0	14.1

[1] Studies in this laboratory on normal growing, young monkeys showed a normal range in hemoglobin concentration of 14–15.5 gm %.

Ten-in-one-ration

The 10-in-1 ration, when fed in the unsupplemented form, produced a slight gain in weight and maintained monkeys for at least 2 months. At the end of this period, there was a sharp decline in weight. The addition of a mixture of the water-soluble vitamins brought about a weight response, but the maximum weight was not greater than the previous maximum attained. The weight did not rise above this point, but was maintained if the vitamin addition to the diet was continued (fig. 1 — monkey no. 293).

With the addition of 100 ml of raw milk per day, the weight of the monkeys continued to increase at a normal rate. If this addition was delayed for 1 month, it took some time before this weight response was noted. The 10-in-1 ration plus these additional supplements supported good growth in monkeys for over a year. When the milk was withdrawn, the gain in weight continued for approximately 2 months. At the end of this period, there was a slow but constant decline in weight (fig. 1 — monkey no. 294). The appearance of the 5 monkeys on this regimen was quite normal at the end of the experimental period.

The hemoglobin concentration of the blood of monkeys on this diet was always below normal. The addition of raw milk or vitamins did not produce any significant variation in this respect. After 1 year, with both supplements being fed in addition to the 10-in-1 ration, the hemoglobin concentration was approximately 12.0 gm %. The differential leucocyte count was normal throughout the experiment.

K ration

When 9 young rhesus monkeys were fed K ration, there was practically no gain in weight. They were able to maintain themselves on this ration for approximately 2 months. At the end of this period, anorexia developed. Feeding the animals by stomach tube did not alleviate the condition. If supplementation was not started quickly enough, the animals lost weight rapidly and death followed.

When monkeys fed the K ration started to lose weight, a mixture of all the water-soluble vitamins was added as a supplement. The addition produced an immediate weight response. The weight increased at a slow but constant rate for a period of 9 months (fig. 1 — monkey no. 305). When 100 ml of raw milk per day was added to the vitamin supplement, a growth response and a rapid rate of gain were noted. This normal rate of gain lasted over 1 year when both supplements were fed (fig. 1 — monkey no. 289).

The data indicated that of the 3 combat rations tested, K ration was the poorest in supporting growth of monkeys (fig. 2). It was decided to concentrate on this ration to find exactly which factors were producing a response when added to the diet. Monkeys were placed on the unsupplemented ration for about 6 weeks. At that time, they were supplemented with individual vitamins, while the weight and hemoglobin responses were closely followed.

The supplementation procedure consisted of feeding a single vitamin at a time. This was done with animals which had not gained weight for at least a month previously, or were

losing weight rapidly. A response was considered significant if the monkey gained at least 100 gm in 7 to 14 days. Once a vitamin was added to the diet, it was usually fed daily thereafter. Another vitamin was not added to the diet until a definite plateau in the weight curve or a rapid loss of weight was noted.

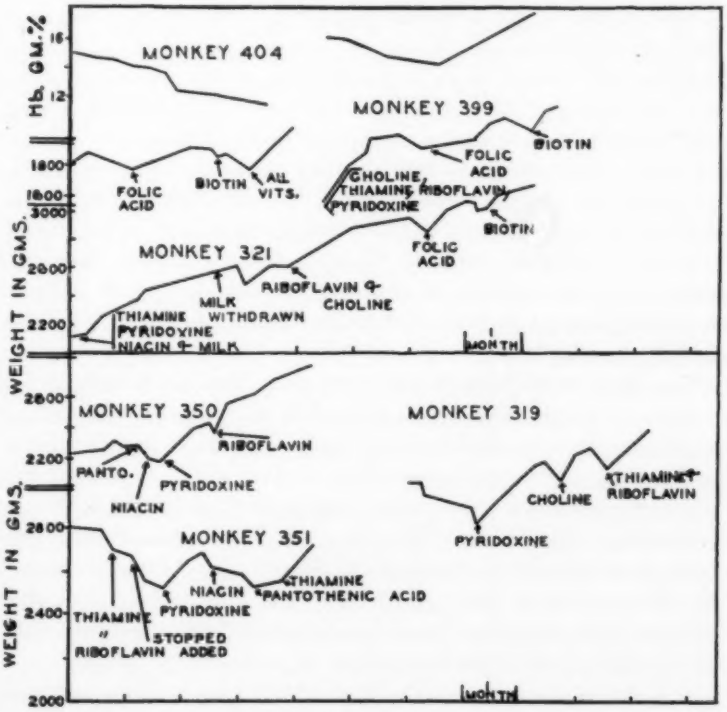

Fig. 2 Typical growth responses of monkeys fed the K ration. Individual vitamin supplementation is shown in various animals.

When pyridoxine was added singly as a supplement to the ration, a sharp weight response was noted. Identical responses were noted in at least 4 different monkeys when pyridoxine was added before or after other vitamins. The weight re-

sponse lasted a short time, and then a loss of weight followed. This type of response occurred with all vitamins active in stimulating growth on K ration.

A mixture of thiamine and riboflavin was added to the diet of monkeys that were losing weight following the original response on B_6. There was an immediate gain in weight. In other cases, when thiamine or riboflavin was added separately to the vitamin supplement following pyridoxine therapy, there was a similar significant response. When either of these vitamins was added prior to pyridoxine, no increase in weight could be detected following such addition. This indicated that pyridoxine was a limiting factor in these studies.

Choline was added to the vitamin supplement of monkeys following the typical response to pyridoxine. In all cases this addition brought about a definite weight increase. It was tried prior to pyridoxine therapy, and results indicate that the same response could not be observed. It was necessary to have added B_6 present in the diet in order to obtain activity by the addition of choline.

The same results were obtained with biotin. A weight increase was produced by its addition to the diet, but only when supplementary pyridoxine was present. This substantiates the limiting action of pyridoxine.

Niacin and pantothenic acid were added as individual supplements to this ration. They were fed before and after the monkeys received other vitamins. In all cases, little response was detected upon their addition to the diet. Pyridoxine did not alter this condition in contrast to its effect when thiamine and riboflavin were the supplements.

In 1 particular case, a monkey which had received unsupplemented K ration for approximately 2 months was fed milk when anorexia developed. It was necessary to give him a single dose of B_6 (2 mg) in order to prevent further loss of weight and possible death. The milk supplement alone supported a slow rate of gain. The addition of thiamine, which is present in milk in small amounts, was able to produce a weight increase. Additional niacin and vitamin C, both low

in milk, had no effect whatsoever. Pyridoxine, even when fed in addition to milk, brought about a very large and prolonged weight increase.

Thiamine, riboflavin and pyridoxine were fed as supplements to monkeys on K ration from the beginning of an experiment. The diet did not support growth of monkeys, but did extend the maintenance period on the ration. On this regimen, monkeys could survive for at least 3 months. In 1 case, the addition of choline at that time brought about a sharp weight increase.

Blood

The hemoglobin concentration of the blood of monkeys on K ration dropped to approximately 10 gm per 100 ml of blood after the animals had been on the unsupplemented ration for 6 weeks. The addition of a mixture of water-soluble vitamins improved this condition and produced an optimum hemoglobin concentration of 15 gm %. The hemoglobin remained at this optimum level when no further additions were made to the ration, and while the gain of weight was slow.

As stated previously, the addition of milk produced a rapid gain in weight. This caused a drop in the hemoglobin concentration of the blood. Monkeys which had been fed both milk and vitamins as supplements to the ration for a period of 1 year showed a hemoglobin concentration of approximately 12.5 gm %.

Pyridoxine and folic acid were the only vitamins tested which produced a response in hemoglobin. The hemoglobin response was slower than the weight response, but an optimum level was reached in a short time. This same type of response was noted in studies on pyridoxine and folic acid deficiencies on a synthetic ration.

Pyridoxine produced an optimum hemoglobin level which lasted approximately 2 months, after which sub-optimum levels were observed. Folic acid was the only vitamin able to bring the hemoglobin back to the normal concentration.

This effect of folic acid could not be observed unless pyridoxine was present as a supplement to the ration.

The differential leucocyte count was normal throughout the experiments.

Autopsy

One monkey died 1½ months after being placed on K ration. The autopsy revealed extensive hemorrhagic lesions in the colon which extended as far as the appendix. Indications of pneumonia complicated the condition.

Another monkey died in about the same length of time on the K ration. Autopsy disclosed a similar hemorrhagic condition in the colon, as well as a mottled liver containing 60% fat (dry weight). The case was complicated with tuberculosis, lesions being easily detectable in the lungs.

A third animal failed after responding to treatment with individual vitamins; pyridoxine, choline, thiamine and riboflavin had been fed in addition to the K ration. Autopsy revealed extensive intestinal introsusception. This was believed to be the cause of death. Aside from a large, dark spleen and an enlarged liver, all other organs appeared normal.

DISCUSSION

The results presented in this paper are based on the *ad libitum* feeding of army combat rations to young growing monkeys. It should be emphasized that the materials used represent only a limited sampling since the items included in the rations have been changed from time to time. Therefore, it must be recognized that the samples taken from other manufacturing sources and at different times might give results varying in some degree from those reported in these experiments. One other important consideration is the fact that in these experiments, growth and hematopoiesis covering an extended period of time were the 2 criteria used in evaluating the several rations. Actually, in the field these foods are consumed for the most part by mature men and for relatively short periods of time.

Data obtained from the feeding experiments indicate that none of the combat rations studied could support growth in the monkey for extended periods of time. The C ration produced the best results, since it was able to support a slight gain in weight when fed in the unsupplemented form. It was only necessary to add a mixture of the water-soluble vitamins in order to improve this ration. When this was done, the diet supported fairly good growth of monkeys for over a year.

The 10-in-1 ration needed 2 supplements in order to improve its quality. The addition of a mixture of the water-soluble vitamins produced a weight increase but not as great as in either of the other cases. However, when raw milk plus the vitamins was added to the diet of monkeys on 10-in-1 ration, good growth was obtained over extended periods.

Monkeys fed the K ration died within 2 months. The addition of a mixture of water-soluble vitamins improved the ration to a certain extent, but raw milk was necessary before it was able to support good growth of monkeys.

Eight of the 11 vitamins present in the mixture of these water-soluble factors were tested individually by adding them as supplements to the K ration. It was observed that a significant increase in weight was obtained when either thiamine, riboflavin, pyridoxine, choline, folic acid or biotin was added to the diet. Niacin and pantothenic acid were inactive in this respect.

This particular study is one of the first instances in which monkeys have been fed a ration which seems to be deficient in a number of factors. The type of response observed is similar to that which is obtained with rats on a multiple-deficient diet. A response in weight is noted when a factor is fed in which the diet is deficient, but the weight drops soon thereafter. It is difficult to state whether a response indicates a lack of the factor added, or is an indirect effect of the addition.

The Committee on Food Composition of the Food and Nutrition Board, National Research Council, reported the

analysis of the K ration. Their reported analysis, as well as the recommended allowances are presented:

	N.R.C. ANALYSIS	DAILY ALLOWANCE RECOMMENDED FOR MODERATELY ACTIVE MAN BY N.R.C.
	mg/day unit	*mg*
Thiamine	1.33	1.5
Riboflavin	2.60	2.0
Niacin	12.7	15.0

From these figures it is seen that the levels of thiamine and riboflavin in the ration should be sufficient to satisfy the requirements of man. However, the results show that these vitamins produced a growth response when added to the ration fed the monkeys.

The National Research Council allowances are usually high enough to be optimum under most conditions. The requirements for thiamine and riboflavin appear to be the same for man and monkey, when figured on the basis of body weight. It must be remembered that the figures in the table represent a daily portion for man, whereas the monkeys were fed *ad libitum*.

It is possible that the levels of vitamins needed on this ration are higher than will suffice ordinarily. It has been shown that the type of carbohydrate, protein or fat may alter the amount of the known vitamins necessary on a particular diet (Luckey et al., '46). Then again, there may be an imbalance of factors in the ration. This has been shown to have an adverse effect in the case of amino acids (Krehl et al., '46).

It is difficult to believe that the growth responses noted on the addition of thiamine or riboflavin are due to the fact that the K ration is deficient in these vitamins. The lack of any specific deficiency signs supports this contention. The freckled dermatitis which is typical of a riboflavin deficiency in the monkey was completely absent. No symptoms of thiamine deficiency as described by Waisman and McCall ('44) could be observed.

The possibility of stimulation of intestinal synthesis is plausible. The addition of these known factors may cause the intestinal flora to produce known or unknown factors in which the ration is low. This would explain the growth response in spite of the fact that the vitamins added were present in the ration in optimum amounts.

The question of poor absorption was eliminated by a study of urinary excretion. The urines of monkeys on K ration receiving added riboflavin and riboflavin plus milk were compared. Greater amounts were secreted in the second case. The fact that an appreciable amount of riboflavin was present in the urine, eliminated poor absorption on this ration as a significant factor.

An interesting observation in these experiments is that pyridoxine is a limiting factor. This vitamin must be present as a supplement to the diet before the effect of 3 of the 4 active vitamins can be noted; it does not fall into the same position as thiamine and riboflavin. Microbiological assays for vitamin B_6 (Rabinowitz and Snell, '47) indicated 0.185 mg per 100 gm of ration. Though the B_6 requirement of the monkey is not definitely known, experience with other animals indicates that this may be a border-line amount.

There seems to be a clear-cut deficiency for folic acid and pyridoxine. The increase in hemoglobin concentration upon the addition of the water-soluble vitamins was due to pyridoxine and folic acid. This was proven with individual vitamin supplementation. They were the only vitamins able to cure deficiency symptoms other than growth. Here again, pyridoxine showed its limiting effect. Folic acid was ineffective in producing a hemoglobin response unless B_6 was present as a supplement to the diet.

Biotin seems to fall into the same category as thiamine and riboflavin. Its beneficial effect is noted only in the presence of supplementary pyridoxine. Though several monkeys developed a loss of hair previous to complete vitamin supplementation, biotin could not be definitely identified as the

curative agent. Its effect may be due to stimulation rather than to an actual deficiency for the vitamin.

In considering choline, it is difficult to determine exactly how this vitamin may be concerned. It was added originally when a fatty liver was noted upon autopsy of 1 monkey which died on the unsupplemented K ration. Choline produced a significant weight response when added to the diet of monkeys. No data are available on the choline content of the ration, and no work has been performed with choline deficiency in monkeys. It may very well be a true vitamin deficiency in the case of this factor.

Intestinal introsusception has been reported in dogs on a pantothenic acid-deficient diet (McKibbin et al., '40). It was also detected on autopsy of a monkey on K ration, one of the few times this has been observed in the history of the monkey colony at this laboratory. Pantothenic acid was added as a supplement to the ration of other monkeys after this observation was noted, but results indicate that it had no effect.

Niacin proved of little value as a supplement to the K ration. Workers at this laboratory (Cooperman et al., '46b) were unable to produce a true niacin deficiency in monkeys fed a purified ration. The other water-soluble vitamins were not studied extensively enough with the K ration to report on them at this time.

Thiamine, riboflavin and pyridoxine were added as supplements to K ration from the start of an experiment. This extended the maintenance period on this ration for at least another month. It supports the evidence that one of these factors is deficient in the K ration.

The blood picture is an indication of the adequacy of a ration. The hemoglobin concentration of the blood of monkeys on the C ration was within the normal range of 14 to 15.5%, when this ration was supplemented with vitamins. Even with vitamins plus milk, the 10-in-1 ration was not able to support a normal hemoglobin level. There is still a factor lacking in the case of this ration. Evidence indicates that it is not the same factor as reported by Cooperman et al. ('46a), as the

characteristic reversal of the neutrophil-lymphocyte count was not observed.

The K ration, with additional vitamins, supported a normal hemoglobin level when the monkeys grew at a slow rate. When the growth rate was normal, the hemoglobin concentration dropped to a sub-optimal level. Here again, differential leucocyte counts showed that this was not due to a deficiency of the monkey anti-anemia factor.

It must be realized that man and monkey do differ in their nutritional requirements. Clear-cut examples of pyridoxine, pantothenic acid and biotin deficiencies have not been observed in man. These have been produced and studied quite intensively in the monkey. Biotin deficiency, which usually requires the administration of raw egg white or sulfa drug before symptoms are observed, can be produced in monkeys simply by eliminating biotin from the ration. This evidence tends to interfere with correlation between the species.

The monkeys used in these experiments were young animals with little stores of essential nutrients. These fast-growing animals have a higher requirement for such nutrients than would be needed for maintenance. These facts indicate that the rations cannot be rejected on the basis of our experiments. They have been used to maintain healthy men for short periods. Under such conditions, the rations would probably be quite sufficient. If the rations were to be used to support growth or for maintaining depleted individuals, certain improvements would be advisable.

SUMMARY

The army combat rations K, C, and 10-in-1 were fed *ad libitum* to young growing monkeys. The materials fed represent only a limited sampling since the items included in the rations have been changed from time to time. Therefore, the samples taken from other manufacturing sources and at different times might give results varying in some degree from those reported in this instance. In these experiments, growth

and hematopoiesis covering an extended period of time were the 2 criteria used in evaluating the several rations.

The results of the experiments carried out under these conditions indicate that none of the combat rations tested could support growth in monkeys. The C ration could be substantially improved by the addition of a mixture of the water-soluble vitamins. The 10-in-1 ration produced normal growth in monkeys when supplemented with the vitamins plus raw whole milk. The K ration required the same supplements in order to support normal growth in monkeys for an extended period of time.

The supplemented C and K rations could support a normal hemoglobin concentration. Normal growing monkeys on the supplemented K and 10-in-1 ration had sub-optimum hemoglobin levels.

Thiamine, riboflavin, pyridoxine, folic acid, biotin and choline were individually active in stimulating growth in monkeys on the K ration. Niacin and pantothenic acid were inactive in this respect. The effect of thiamine, riboflavin and biotin may be indirect, since specific deficiency symptoms are absent. For the growing monkey, K ration seems deficient in pyridoxine and folic acid, and possibly choline as well.

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THE SYNTHESIS OF CERTAIN B VITAMINS BY THE RABBIT[1]

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The physiological significance of the B vitamins has been intensively studied in many animal species and it is generally accepted that the mono-gastric animals require a dietary source of most of the B vitamins, while the functional ruminants synthesize them in their digestive tracts. On the other hand, very little work has been done to determine the role of the different B vitamins in the nutrition of the rabbit.

The first indications of a very particular behavior of rabbits in relation to their requirement of a vitamin of the B complex were reported by Passmore ('35). A purified diet supplemented with a yeast preparation was modified to produce a vitamin B_1 deficiency by autoclaving the yeast preparation at 130°C. for 20 minutes at pH 9.5. Three rabbits fed this diet for 40 days gained in weight and it was concluded that vitamin B_1 was synthesized in the alimentary canal. Baglioni ('36) fed 2 rabbits a diet deficient in vitamin B_1, which consisted of an autoclaved mixture of beans and butter supplemented with calcium lactate, sodium chloride and lemon juice. The animals did not show any symptoms attributable to a vitamin deficiency after ingesting the diet for 65 days, and the urine contained sufficient vitamin B_1 to prevent beriberi in pigeons receiving a polished rice diet. Hogan and

[1] Some of the data were taken from a thesis submitted to the Graduate School by Orlando Olcese in partial fulfillment of the requirements for the degree of Master of Science.

Ritchie ('34) succeeded in raising rabbits when a diet of a semi-purified nature was fed. In a later report (Hogan and Hamilton, '42) it was shown that rabbits grew at the normal rate on simplified rations which contained dried yeast as the source of all water-soluble vitamins.

Wooley and Sebrell ('44) reported that rabbits required niacin for normal growth when fed a purified diet which contained 20% casein. In view of the recent work on the apparent utilization of dietary tryptophane as a source of nicotinic acid, the report of these workers, that an increased growth rate was observed when nicotinic acid was added to a purified diet, is rather surprising. This seems particularly true when little or no growth response was obtained with the rat when nicotinic acid was added to diets also containing 20% casein (Hundley, '47; Schweigert and Pearson, '48).

It was of importance, therefore, to obtain additional data on the requirements of the rabbit for B vitamins. The rate of growth and urinary and fecal excretion of riboflavin and pantothenic acid were determined when diets low in these vitamins were fed. Some studies have also been carried out on the excretion of biotin and pteroylglutamic acid (folic acid) by the rabbit.

EXPERIMENTAL

Weanling rabbits of the New Zealand White breed were used for these experiments, and animals of the same sex were equally distributed among the different groups in each experiment. The animals were kept in groups in wire bottom cages and were weighed at weekly intervals.

The composition of the different diets used is shown in table 1. During the first few days on experiment, the simplified diets were mixed with stock ration to facilitate the acceptance of the purified rations. After this period the rabbits accepted the purified diets without any objection. Food and water were provided *ad libitum*.

Metabolism studies were carried out to determine whether or not pantothenic acid and riboflavin were being synthesized by the rabbit. Since the diets did not contain additional biotin

or pteroylglutamic acid, it seemed advisable to determine the balance of these 2 vitamins. Food consumption records were kept and the amounts of pantothenic acid, riboflavin, biotin and folic acid in the diets determined.

TABLE 1

Composition of the diets.

DIET NO.	1	1a	2	2a	3	4
	gm	*gm*	*gm*	*gm*	*gm*	*gm*
Dextrin	37.5		37.5		37.5	
Cerelose [1]		37.5		37.5		37.5
Beet pulp	30	30	30	30	30	30
Cellulose	3	3	3	3	3	3
Peanut oil	8		8		8	
Corn oil [2]		8		8		8
Commercial casein	18		18		18	
Purified casein [3]		18		18		18
A and D oil	0.5	0.5	0.5	0.5	0.5	0.5
Salts IV [4]	3	3	3	3	3	3
	Supplements per 100 gm of diet					
	mg	*mg*	*mg*	*mg*	*mg*	*mg*
Mixed tocopherols	50	50	50	50	50	50
Choline chloride	200	200	200	200	200	200
Niacin	20	20	20	20	20	20
Inositol	10	10	10	10	10	10
2-methyl 1,4-naphthoquinone	0.075	0.075	0.075	0.075	0.075	0.075
Pyridoxine	0.7	0.7	0.7	0.7	0.7	0.7
Thiamine	0.7	0.7	0.7	0.7	0.7	0.7
Riboflavin	0.7	0.7	0.7	0.7	0.7	
Calcium pantothenate			1.5	1.5	0.5	1.5

[1] Glucose monohydrate.

[2] Mazola.

[3] Labco.

[4] Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E.B., J. Biol. Chem., *138:* 459, 1941.

For the metabolism studies, the rabbits were placed in individual wire bottom metabolism cages for a period of 72 hours; the feces were retained by a screen, while the urine passed through and was collected in dark bottles containing acetic

acid. These bottles were changed every day and kept in a refrigerator, and at the end of the third day the contents of the 3 bottles from each rabbit were mixed together. The volume of urine collected during the 3-day periods ranged from 150 to 350 ml; however, water was sometimes spilled from the drinking containers and the volume collected was therefore increased. The urine collected from each rabbit in each period was made up to a volume of 1000 ml with water to give a more uniform concentration of the vitamins. The urine samples were neutralized and appropriate aliquots were taken for assay.

The feces, after being collected, were dried for 24 hours at 60°C., weighed, and finely ground. Samples were then taken for determinations of pantothenic acid, riboflavin and biotin.

Pantothenic acid was determined by the method of Neal and Strong ('43) after liberation of the vitamin by autoclaving and enzymatic digestion followed by filtration at pH 4.6. A slight modification was made in the medium by replacing the Vitab supplement with synthetic vitamins. Riboflavin was determined by the method of Snell and Strong ('39). The vitamin was liberated by autoclaving the samples with 0.1 N HCl, and after cooling, the samples were filtered at pH 4.6. Biotin was determined with *Lactobacillus arabinosus* as the test organism by the method described by Wright and Skeggs ('44). The fecal samples were autoclaved with 6 N H_2SO_4 for 2 hours to liberate the vitamin. Folic acid was determined by the method of Teply and Elvehjem ('45) with *S. faecalis R* as the test organism.

RESULTS AND DISCUSSION

In order to determine the performance of rabbits fed different levels of pantothenic acid, the first experiment was carried out with 3 groups of 6 rabbits each receiving diets which contained additions of 0, 0.5, and 1.5 mg of Ca pantothenate per 100 gm of feed (diets 1, 3 and 2, respectively, table 2). During the 50 days on experiment the animals grew normally and no symptoms of a vitamin deficiency were ob-

served in the rabbits fed the pantothenic acid-low diet. The average gains for the 50-day period were similar, 1292, 1227 and 1266 gm, respectively, for the 3 groups. These results indicated that dietary pantothenic acid is not required by the rabbit and that the small differences in the growth observed among the 3 groups were not due to dietary treatment, but to variance within the same population.

Since evidence of a vitamin deficiency was not produced in the first experiment, another series of experiments was conducted to obtain further information. Two groups of 5 rabbits each were used. One group was fed a pantothenic acid

TABLE 2

Effect of dietary treatment on the growth of rabbits.

GROUP	NUMBER OF RABBITS	DIET	DAYS ON EXPERIMENT	BODY WEIGHT GAIN
				gm
1	6	1	49	1292
2	6	2	49	1266
3	6	3	49	1227
4	5	1a	112	2117
5	5	4	112	2164
6	5	1a	105	2235
7	5	2	105	1850
8	5	4	105	1847

deficient diet (no. 1a), and the other, a riboflavin deficient diet (no. 4). More highly purified ingredients were used in order to reduce the amount of vitamins contributed by the diets fed. The pantothenic acid content of the pantothenic acid deficient diet was found to be 0.31 μg per gram. The riboflavin content of the riboflavin deficient diet was 0.33 μg per gram. Diets this low in pantothenic acid and riboflavin would not support normal growth in rats. Unna ('40) and Henderson et al. ('42) have shown that 80 μg of pantothenic acid is the minimum daily requirement of the young rat. Assuming a daily consumption of 20 gm of feed by the rat, diet 1a would provide only 6.2 μg of pantothenic acid. Wagner

et al. ('40) and Mannering et al. ('41) reported that the minimum daily requirement of riboflavin for the rat is between 18 and 30 μg. With 20 gm of daily food consumption, diet 4 would provide only 6.6 μg of riboflavin per day.

This experiment was carried out during the summer time and the hot weather was not favorable for optimum performances. Consequently, the animals had a low food intake; however, the ingestion of the diets low in pantothenic acid and riboflavin failed to produce any vitamin deficiency symptoms. After 55 days, one of the rabbits on the pantothenic acid deficient diet developed diarrhea and died. This animal had been gaining weight normally and its death is not attributed to a pantothenic acid deficiency. The growth of the other animals in both groups was satisfactory. The rabbits were fed these diets for 16 weeks. At this time the average weight of the rabbits on the pantothenic acid deficient diet was 3196 gm and the average weight of the rabbits on the riboflavin deficient diet was 3130 gm.

Starting with the sixth week, some of the rabbits tended to eat the hair of the other animals. Areas devoid of hair appeared on some of them, apparently not attributable to spontaneous alopecia. This was evinced by the growth of new hair when the rabbits were placed in individual cages.

At the end of the 16-week period the animals were changed to rations containing 1% sulfasuxidine. The sulfasuxidine was added at the expense of the cerelose. While on this dietary regimen, 2 of the rabbits in each group developed diarrhea and died. None of the animals showed any symptoms that could be definitely attributed to a pantothenic acid or riboflavin deficiency. Low gains were obtained after the addition of sulfasuxidine to the diets, due in part to the fact that the rabbits were reaching maturity. However, 2 rabbits on the low pantothenic acid diet and 1 on the low riboflavin diet showed a loss of weight.

Urine and fecal collections were made for 3 different periods for each rabbit before and after the feeding of sulfasuxidine. The results obtained for the metabolism

studies are summarized in table 3. Each figure for the pantothenic acid deficient rabbits is the average obtained for 3 collections from each of 4 rabbits; for the riboflavin deficient animals each figure is the average for 3 collections from each of 5 rabbits. The data obtained on the amount of riboflavin excreted demonstrate clearly that this vitamin is being synthesized by the rabbit. The animals fed the riboflavin deficient diet ingested an average of 11.1 μg of riboflavin daily and excreted 127.1 μg before the feeding of sulfasuxidine. This

TABLE 3

Ingestion and excretion of riboflavin, pantothenic acid and biotin by rabbits when fed low-pantothenic acid and low-riboflavin diets before and after the addition of sulfasuxidine (values expressed in μg per 24 hours).

DIET NUMBER	DESCRIPTION	BEFORE FEEDING SULFASUXIDINE				AFTER FEEDING SULFASUXIDINE			
		Intake	Excretion			Intake	Excretion		
			Urine	Feces	Total		Urine	Feces	Total
		Riboflavin balance							
4	low-riboflavin	11.1	112.9	14.2	127.1	11.0	109.6	31.0	140.6
		Pantothenic acid balance							
1a	low-pantothenic acid	8.5	34.7	15.2	49.9	8.8	28.0	27.6	55.6
		Biotin balance							
4	low-riboflavin	0.27	1.64	..	..	0.33	1.08	..	..
1a	low-pantothenic acid	0.28	0.99	..	..	0.29	0.75	..	..

represents an 11-fold increase in the amount excreted as compared to the amount ingested. Similar results were obtained for the pantothenic acid studies. For the group receiving the low pantothenic acid diet the excretion of this vitamin was 49.9 μg per day as compared with an ingestion of 8.5 μg. The amount excreted was, therefore, 6 times higher than the amount ingested. Since biotin assays were not made of all the fecal samples, the data obtained for only the urine are presented in the table. However, the urine values alone indi-

cate that biotin was synthesized. The daily ingestion of biotin by the rabbits fed diets 1a and 4 averaged 0.28 and 0.27 μg, with corresponding excretions in the urine of 0.99 and 1.64 μg, respectively. The daily ingestion of folic acid by the rabbits fed diets 1a and 4 averaged 1.1 μg. The average daily excretion of this vitamin in the urine by the 5 rabbits receiving diet 1a was 30.6 μg, and by the 5 receiving diet 4, 34.1 μg, which greatly exceeded the intake.

After the feeding of sulfasuxidine, there was a slight increase in the amount of riboflavin excreted by the rabbits fed the low riboflavin diet, as compared with the excretion before the feeding of the sulfa drug. The amount of pantothenic acid excreted by the rabbits fed the low pantothenic acid diet after the feeding of sulfasuxidine also increased slightly as compared to the amount excreted before the feeding of the drug. Similarly the excretion of biotin was not reduced when sulfasuxidine was fed (table 3). The ingestion of this level of sulfasuxidine did not, therefore, appear to have affected the amounts of these vitamins synthesized by the rabbit.

The excretion of folic acid, however, showed a marked decrease when sulfasuxidine was fed. After the feeding of the sulfa drug, the daily urinary excretion of folic acid was reduced from an average of 30.6 to 1.30 μg for the animals fed diet 1a, and from 34.1 to 0.83 μg for the ones fed diet 4. Several of the rabbits died after developing a severe diarrhea when fed sulfasuxidine. All these facts suggest that if a deficiency of a vitamin was responsible for the deaths, it must have been a deficiency of folic acid or/and other factors(s).

A third series of experiments was carried out in order to compare the growth of 5 rabbits fed a diet low in pantothenic acid and 5 fed a diet low in riboflavin, with the growth of a control group receiving both of the vitamins. They received diets 1a, 4 and 2a, respectively. The animals were kept on these diets for 15 weeks, and none of them showed any symptom either of a vitamin deficiency or diarrhea. The gains were good with all 3 diets and the group fed the low-pantothenic acid diet made somewhat better gains than the other two. It

is of interest to point out that after termination of these experiments, 2 of the does fed the riboflavin-low diet produced young, thereby indicating that not only will these diets support growth but reproduction as well.

Metabolism studies were carried out also with these rabbits and the excretion of pantothenic acid, riboflavin and biotin determined. The purpose was to verify the results of the first studies and at the same time to compare the ingestions and

TABLE 4

Ingestion and excretion of riboflavin, pantothenic acid and biotin by rabbits receiving low-pantothenic acid, control and low-riboflavin diets (values expressed in μg per 24 hours).

GROUP	DIETARY TREATMENT	INTAKE	EXCRETION		
			Urine	Feces	Total
		Riboflavin balance			
6	low-pantothenic acid	407.3	419.5	36.0	455.5
7	control	416.3	365.7	33.2	398.9
8	low-riboflavin	17.0	239.3	25.8	265.1
		Pantothenic acid balance			
6	low-pantothenic acid	17.2	99.8	45.1	144.9
7	control	869.4	425.2	37.6	462.8
8	low-riboflavin	790.5	449.4	36.2	485.6
		Biotin balance			
6	low-pantothenic acid	0.56	2.14	1.35	3.49
7	control	0.57	2.71	1.55	4.26
8	low-riboflavin	0.52	1.96	1.23	3.19

excretions of the rabbits fed the low-riboflavin and low-pantothenic acid diets with the ingestions and excretions of the control group. The collections were made during the fall season of the year and, as might be expected, the ingestion and excretion were much higher than in the second series conducted during the summer season. Collections were made beginning with the fifth week through the eighth week of the experimental feeding periods.

The results of this study are summarized in table 4, each figure representing an average of 15 collections (3 collections for

each of 5 rabbits). The amounts of the vitamins excreted did not vary appreciably for each of the 3 collections made; consequently, only the average for all collections is presented. The average daily excretions of riboflavin were: for the animals fed the low-pantothenic acid diet, 455.5 μg; for those fed the control diet, 398.9 μg; and for the ones fed the low-riboflavin diet, 265.1 μg, with corresponding riboflavin intakes of 407.3, 416.3 and 17.0 μg per day, respectively. These data show, therefore, that a high amount of synthesis occurred when the low-riboflavin diet was ingested. For the other 2 groups fed additional riboflavin, the excretion approximated the intake.

The average daily excretions of pantothenic acid were: for the rabbits receiving the low-pantothenic acid diet, 144.9 μg; for the rabbits receiving the control diet, 462.8 μg; and for the ones receiving the low-riboflavin diet, 485.6 μg, with corresponding intakes of 17.2, 869.4 and 790.5 μg, respectively. There was a significant synthesis of pantothenic acid by the rabbits fed the low-pantothenic acid diet. The previous findings, therefore, were corroborated with these studies. Proportionately more riboflavin was synthesized than pantothenic acid, as in the previous study.

The total biotin excreted per day in the case of the animals fed the low-pantothenic acid diet was 3.49 μg as compared to an intake of 0.56. For the control groups, the total biotin excreted amounted to 4.26 μg per day with an intake of 0.57, and for the group fed the low-riboflavin diet the total excretion was 3.19 μg, with an intake of 0.52.

The fact that a high degree of riboflavin and pantothenic acid synthesis was observed when the diets low in these respective vitamins were fed, while the excretion approximated the intake when additional vitamin supplements were included in the diet, suggests either that the best conditions for the synthesis of the vitamins are met when the ration is low in those particular vitamins or that they are destroyed when fed in high amounts. This fact is not surprising since

destruction of riboflavin occurred when it was fed in large amounts to ruminants (Pearson and Schweigert, '47).

The data presented in this work indicate that growing rabbits either have a very low requirement or do not require a dietary source of either pantothenic acid or riboflavin. As for the mechanism by which the absorption of the synthesized vitamin takes place, there are 2 possibilities. The first is that the vitamins synthesized by the flora of the intestinal tract are absorbed directly from the cecum and colon. The second possibility is that, due to the normal night coprophagy shown by the rabbit, the vitamins synthesized in the cecum are absorbed in the duodenum after the ingestion of the feces. It has been demonstrated that rabbits reconsume the feces as voided from the body (Morot, 1882; Madsen, '39; Taylor, '39, '40; and Eden, '40). Taylor ('39) has shown that by the normal function of night coprophagy in the rabbit, very large amounts of feces are reingested. He found that between one-third and one-half of the stomach contents is normally of fecal origin. The suggestion that coprophagy serves as a means of facilitating absorption of the vitamins synthesized in the intestinal tract is supported by the observations of several investigators (Steenbock et al., '23; Dutcher and Francis, '24; Smith et al., '25; Cowgill et al., '25; Salmon, '25; and Kennedy and Palmer, '28) that rats on B vitamin deficient diets showed improved growth when allowed access to their own feces. Although the rabbits used in the present study were kept in cages fitted with wire screened bottoms, no special techniques were employed to prevent coprophagy.

Although dextrin was used in the first experiments and cerelose in subsequent ones, no direct evidence for the comparative effect of these carbohydrates on the amount of synthesis of B vitamins was obtained since no balance studies were carried out when the dextrin diets were used. Dextrin apparently is more favorable than are the simpler carbohydrates for the synthesis of B vitamins by the rat (Guerrant and Dutcher, '34; Mannering et al., '44; Schweigert et al., '45); however, a deficiency of neither pantothenic

acid nor riboflavin was produced in the present study when cerelose was used as the main carbohydrate source. The inclusion of beet pulp may have favored conditions for vitamin synthesis. Nielsen and Elvehjem ('42) have shown that the ingestion of low-riboflavin and low-pantothenic acid diets is not favorable for the synthesis of biotin. It is apparent, therefore, from the present work that the rabbit is capable of synthesizing large amounts of at least certain B vitamins.

SUMMARY

1. Pantothenic acid and riboflavin are not essential dietary constituents for the growing rabbit. Diets containing as low as 0.31 μg of pantothenic acid and 0.33 μg of riboflavin per gram induced normal growth in rabbits. No symptoms of a vitamin deficiency were shown by the animals after ingestion of these diets for 112 days.

2. When balance studies were carried out with 2 groups of animals fed the low-riboflavin diet with a daily intake of 11.1 and 17.0 μg, the corresponding total excretions were 127.1 and 265.1 μg of riboflavin per day. Two groups of rabbits fed a pantothenic acid deficient diet ingested daily 8.5 and 17.2 μg of pantothenic acid, and excreted 49.9 and 145.0 μg, respectively.

3. Data obtained on the ingestion and excretion of biotin and folic acid indicate that these vitamins are also synthesized in this species.

4. When 1% of sulfasuxidine was added to the diet, no symptoms specific for a pantothenic acid or riboflavin deficiency appeared, but 5 out of 10 rabbits developed diarrhea and died.

5. Balance studies showed that with the addition of the sulfasuxidine to the diet, only the excretion of folic acid was decreased to a significant extent.

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MINERAL METABOLISM STUDIES IN DAIRY CATTLE

III. MANGANESE METABOLISM IN THE LACTATING BOVINE [1]

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ONE FIGURE

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Studies dealing with the nutritional significance and the metabolism of manganese in the bovine have been impeded by the difficulty encountered in preparing basal diets sufficiently low in this element.

Although the liver and kidneys appear to be the main sites of manganese storage (von Oettingen, '35), and ingested manganese effects a temporary rise in the blood level which rapidly returns to normal (Reiman and Minot, '20), most of the manganese ingested by animals appears to be excreted. Kobert (1883), Cahn (1884), Harnack and Schrieber ('01), Bargero ('06), Reiman and Minot ('20), Greenberg et al. ('43), and Skinner and Peterson ('30) have demonstrated that ingested manganese is almost entirely excreted in the feces. Several reports indicate that small quantities of the ingested element are absorbed into the blood, but are rapidly lost via

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the liver and bile (Bargero, '06; Reiman and Minot, '20; Richards, '30; Greenberg et al., '43). Only extremely minute quantities of manganese have been detected in the urine (Kobert, 1883; Reiman and Minot, '20; Greenberg et al., '43).

Very low levels of manganese were found in cows' milk: 0.000004% (Richards, '30); 0.000003% (McHargue, '24); and 0.000002% (Archibald, '41; Archibald and Lindquist, '43); however, Archibald and Lindquist ('43) were able to increase the amount 2.5-fold by feeding $MnSO_4$ and their results indicated that the cow may store a reserve of this element during the pasture season. Kemmerer and Todd ('31), using similar treatment, were unable to affect the manganese content of cow and goat milk. The poor-quality semen and lowered fertility of bulls receiving a low manganese ration were improved by supplementing the diet with this element (Lardy et al., '42).

EXPERIMENTAL

The data on manganese metabolism reported here were derived from the balance trials reported previously (Reid et al., '47). Three weekly balance trials were conducted during the first 5 months of lactation at regular intervals with each of 8 Holstein and 4 Guernsey cows, comprising 4 feed groups.

The general feeding plan provided 1 lb. of timothy-clover hay and 3.5 lb. of corn silage per 100 lb. body weight daily and concentrates in sufficient quantity to provide 110% of the Morrison feeding standards ('39). The 4 groups received the following: group I, the basal concentrate mixture (consisting of ground corn, 30 parts; rolled oats, 30 parts; linseed meal, 28 parts; wheat bran, 11 parts and NaCl, 1 part); group II, basal concentrate mixture plus 3% of $CaCO_3$ (c.p.); group III, basal concentrate mixture plus 3% of a mixture of $CaCO_3$ (c.p.) and $MnSO_4$ (c.p.) (proportions of Ca and Mn same as in Mico [4]); group IV, basal concentrate mixture plus 3% of Mico.[4]

[4] Mico has the following percentage composition with respect to the elements listed: calcium 33.0, magnesium 2.0, manganese 0.2, iodine 0.045, copper 0.025, zinc 0.01 and cobalt 0.002.

The method used to determine manganese in all materials was essentially that outlined by Willard and Greathouse ('17) with modifications allowing measurement with an Evelyn photoelectric colorimeter. Table 1 shows the mean manganese content of the feeds ingested and the feces excreted by the 4 groups of cows during 9 trials each.

TABLE 1

Mean manganese content of concentrate mixtures, hay, silage and feces during 9 trials (per cent).

	CONCENTRATE FEEDS				MIXED HAY	CORN SILAGE	FECES			
	I	II	III	IV			I	II	III	IV
Mean	.0063	.0058	.0111	.0121	.0066	.0036	.0143	.0136	.0186	.0201
Standard Error	.0000	.0000	.0000	.0001	.0004	.0001	.0002	.0004	.0010	.0008

RESULTS AND DISCUSSION

Figure 1, showing the range of daily quantities of manganese ingested and excreted in the feces, points out the dependence of the amount of manganese excreted in the feces upon the quantity ingested. These data would indicate that lactating cows retain about the same quantity of the ingested manganese, regardless of the amount ingested, over the range 622.4 to 1325.6 mg daily. A correlation coefficient of 0.958 ± 0.014 existed between the intake and fecal excretion of manganese. On the basis of these data, the expected quantity of manganese retained by lactating cows receiving diets similar to those used in this study may be calculated from a known intake by the following equation:

$$Y = 0.92452X - 85.5 \pm 57.4 \text{ mg}$$

where, Y = mg Mn excreted in feces daily

X = mg Mn ingested daily

then, X — Y = mg Mn retained daily

It is not known whether the same relationship holds or whether the same equation may be applied under other experimental conditions. Factors affecting the retention of man-

ganese by cows have not been studied to any great extent. A similar straight line relationship was observed between the intake and fecal excretion of manganese in our calculations of the data presented by Chornock et al. ('42) for rats on high manganese diets.

Since no manganese was found in reasonable quantities of urine and since only traces were detected in the milk of animals receiving supplemental manganese (as previously reported by Archibald, '41, and Archibald and Lindquist, '43),

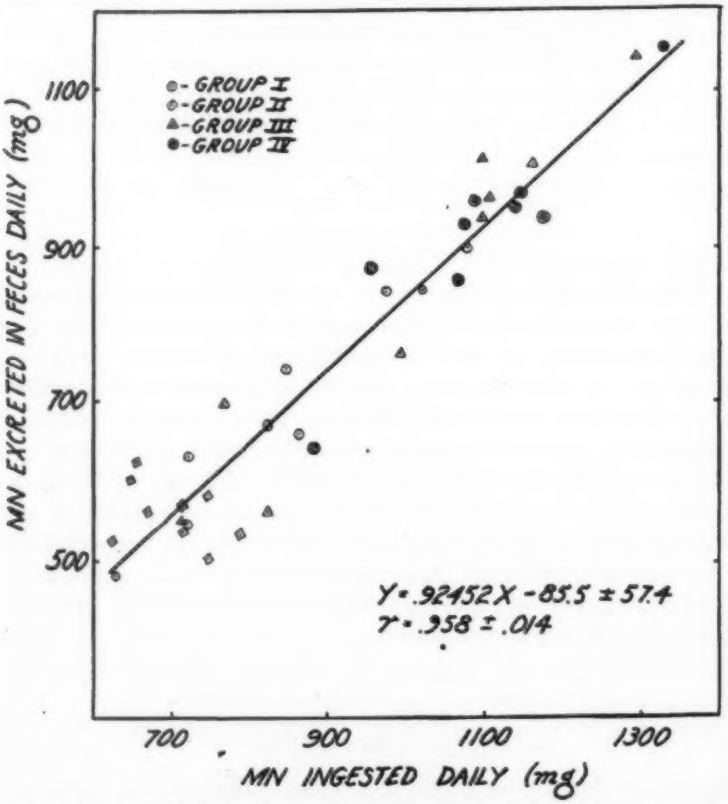

Fig. 1 The relationship of the quantity of manganese ingested to that excreted in the feces.

the quantity of manganese retained daily is practically equivalent to the daily manganese balances for these animals. On the basis of these figures, the occurrence of a negative manganese balance in lactating cows appears remote since it would be extremely difficult to formulate an otherwise normal diet low in manganese for cows. The basal ration employed in this study supplied ample quantities of the element as judged from the great proportion of the intake which was excreted. These data suggested that a lactating cow retains approximately 154.4 ± 9.8 mg of manganese daily.

Manganese added to the rations as $MnSO_4$ appeared to be utilized equally as well as manganese derived from feed stuffs.

The addition of supplemental calcium to the rations of the animals of groups II, III and IV, and of supplemental iodine, magnesium, copper, cobalt, zinc and iron to the ration of group IV cows did not significantly influence the quantity of manganese retained. The means and their standard errors of the amount of manganese used by the groups were: group I, 148.4 ± 12.5; group II, 139.2 ± 26.9; group III, 155.0 ± 20.5 and group IV, 175.0 ± 17.1 mg daily.

SUMMARY

A study was made of the metabolism of manganese in 12 cows at 3 intervals each during the first 5 months of lactation.

Lactating cows retained 154.4 ± 9.8 mg manganese daily; about the same quantity of manganese was used regardless of the quantity of the element ingested. The amount of manganese excreted in the feces was found to be directly proportional to the amount of manganese ingested in the range of 622.4 to 1325.6 mg daily; the correlation coefficient for this relationship was 0.958 ± 0.014. An equation is presented which allows the quantity of manganese excreted daily (and, conversely, the amount retained) to be calculated when the daily intake is known for cows receiving diets similar to those used in this investigation.

The supplementation of the ration with calcium and various trace elements did not appear to influence significantly the amount of the ingested manganese retained.

Manganese added to the ration as $MnSO_4$ was utilized equally as well as that provided by the feed materials.

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INFLUENCE OF NUTRITIONAL FACTORS ON SKELETAL ATROPHY FROM DISUSE AND ON NORMAL BONES OF MATURE RATS[1]

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A previous paper (Armstrong, Knowlton and Gouze, '45) reviewed the problem of skeletal atrophy from disuse and described the effects produced by the administration of an androgenic and of an estrogenic hormone on the composition of the bones of normal and paralyzed limbs of rats. Injections of estradiol dipropionate, at 1 dosage level, were found to result in a reduction of the bony atrophy from disuse and to increase the ash content and amount of bone tissue in the humeri of the normal limbs. Similar studies and results have been presented by Gardner and Halvorsen ('46) who worked with mice. Choline was found by Riley, McCleary and Johnson ('45) not to affect the atrophy of paralyzed limbs of rats, and Landoff ('42) has investigated the atrophy of bones of rabbits caused by immobilization of a limb and by experimentally induced inflammations of the knee joint.

The present study was undertaken in order to obtain data as to the effects of certain nutritional factors on the degree of atrophy occurring in paralyzed limbs. At the same time, an opportunity was also afforded to collect data relative to the influence of the nutritional variations studied on the bones of

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normal limbs of mature animals. There is a wealth of knowledge pertaining to the effects of dietary deficiencies of minerals on the skeleton of young animals, and Sherman and coworkers (Sherman and Booher, '31; Sherman and Sherman, '38; Van Duyne et al., '41; Sherman et al., '41) have investigated extensively the beneficial effects on growth and calcium storage of the feeding, beginning at an early age, of diets more than adequately rich in calcium. There is little published information, however, on the effects produced on the skeleton by nutritional variations imposed on mature subjects aside from the indirect evidence obtained from balance studies. Bauer, Aub and Albright ('29) found that a low intake of calcium resulted in a resorption of the bony trabeculae of the humeri in 4 cats. Incidental to the main purpose of the present study are presented additional data on the effects of estradiol dipropionate on the bones of normal and paralyzed limbs and evidence that sesame oil is not inert in its effects on the skeleton.

METHODS

The Wesson ('32) salt mixture and modifications of this salt mixture as shown in table 1 were used to prepare diets of different calcium, phosphorus and protein contents according to the recipes given in the same table. The ingredients of the salt mixtures for diets 2 and 3 were adjusted so that their content of all minerals, except for a deficiency of calcium or phosphorus, was as close as possible to that of the complete mixture. The Cellu-Flour was added to the deficient salt mixtures in order to equalize on a weight basis the mineral contents common to the 3 salt mixtures.

The dry ingredients of the diets were mixed and the melted fat, into which the liver concentrate and choline hydrochloride were dispersed, was then added and the whole thoroughly stirred in a mechanical dough mixing machine. The diets were prepared in 4 kg batches and stored in the frozen state until used. Table 2 shows the calcium, phosphorus and nitrogen contents of representative batches of diets. The calcium determinations were made by the method of Clark and Collip

TABLE 1

Composition of salt mixtures and diets.

SALT MIXTURES				DIETS		
Ingredient	Complete (Wesson's) (Diets 1, 4, 5, 6)	Calcium deficient (Diet 2)	Phosphorus deficient (Diet 3)		Diets 1–5	Diet 6
	gm	*gm*	*gm*		*gm*	*gm*
NaCl	52.5	77.7	52.5	Sucrose	400	540.5
KCl	60.0	27.8	100.0	Sucrose-vitamin mixture [1]	200	200
KH_2PO_4	155.0	213.8		Lactalbumin (Borden's no. 15–42)	180	
$Ca_3(PO_4)_2$	74.5			Fat (Spry)	169	208.5
$CaCO_3$	105.0		177.1	Salt mixture [2]	40	40
Other ingredients of Wesson salt mixture	102.7	102.7	102.7	Liver concentrate (Wilson's fraction D)	10	10
Cellu-Flour		127.7	117.4	Choline-HCl	1	1
Total	549.7	549.7	549.7	Total	1000 [3]	1000 [4]

[1] Sucrose 1000 gm, thiamine-HCl 10 mg, pyridoxine-HCl 10 mg, riboflavin 15 mg, calcium pantothenate 20 mg.

[2] Complete salt mixture used in diet 1 (control), diet 4 (calcium enriched), diet 5 (phosphorus enriched), and diet 6 (protein deficient); calcium deficient salt mixture used in diet 2, and phosphorus deficient salt mixture used in diet 3.

[3] Plus 6.87 gm $CaCO_3$ in diet 4 (calcium enriched); plus 8.67 gm KH_2PO_4 in diet 5 (phosphorus enriched).

[4] Plus 1.82 gm KH_2PO_4 to substitute for phosphorus present in lactalbumin of diets 1–5.

('25) and the phosphorus analyses by the method of Fiske and Subbarow ('25) applied to solutions of the ash of the diets. Calcium lactate was added as a fixative in the ashing of the calcium deficient diets for phosphorus analysis. Nitrogen was determined by a modification of the method of Ma and Zuazaga ('42).

Male rats of the Sprague-Dawley strain, with weights of 240–250 gm when delivered, were fed commercial dog biscuit

TABLE 2

Analyses of diets.[1]

TYPE OF DIET AND NUMBER	NO. BATCHES ANALYZED	CALCIUM	PHOSPHORUS	NITROGEN
		%	%	%
Adequate: 1	13	0.548 ± 0.0050	0.420 ± 0.0027	2.29 ± 0.016
Calcium deficient: 2	5	0.015 ± 0.0017	0.409 ± 0.0041	2.27 ± 0.011
Phosphorus deficient: 3	4	0.511 ± 0.0123	0.054 ± 0.0026	2.28 ± 0.014
Calcium enriched: 4	2	0.796 and 0.801	0.416 and 0.450	2.29 and 2.33
Phosphorus enriched: 5	2	0.512 and 0.515	0.524 and 0.511	2.33 and 2.31
Protein deficient: 6	4	0.540 ± 0.0028	0.408 ± 0.0192	0.085 ± 0.0038

[1] Data are shown as means ± the standard error of the means except in cases in which all results are shown.

until they attained the weights shown in table 3 when their age was not less than 115 days. In order to permit the development of disuse atrophy of 1 humerus, certain nerves from the cords of the brachial plexus were severed on the right side. Using aseptic technique, the median, radial, ulnar, musculocutaneous, subscapular, axillary, thoracodorsal and suprascapular nerves were exposed by splitting the pectoralis major muscle with a hemostat, and these nerves were severed as they passed through the axilla. The animals of the several

TABLE 3

Treatment and postoperative observations.[1]

GROUP AND NO. OF ANIMALS	TYPE OF DIET AND TREATMENT	WEIGHT AT OPERATION	POSTOPERATIVE GAIN IN WT.	DAILY FOOD CONSUMPTION
		gm.	*gm.*	*gm.*
1 (39)	Operated control diet 1	264 ± 1.8	41 ± 3.4	11.0 ± 0.21
2 (32)	Calcium deficient diet 2	272 ± 1.8	28 ± 3.6	10.8 ± 0.15
3 (26)	Phosphorus deficient diet 3	270 ± 2.0	11 ± 2.5	10.2 ± 0.31
4 (20)	Calcium enriched diet 4	264 ± 0.92	32 ± 2.8	11.2 ± 0.30
5 (20)	Phosphorus enriched diet 5	266 ± 1.1	31 ± 3.0	10.2 ± 0.14
6 (21)	Protein deficient diet 6	268 ± 1.2	— 66 ± 1.5	5.9 ± 0.15
7 (23)	Food restricted [2] diet 1	264 ± 1.4	— 37 ± 1.6	6.0
8 (20)	Unoperated control diet 1	272 ± 1.2	82 ± 3.4	14.8 ± 0.32
9 (23)	Food restricted sesame oil alternate days [3] diet 1	271 ± 1.4	— 7 ± 1.5	8.9 ± 0.03
10 (23)	Estradiol D. P. alternate days diet 1	267 ± 1.2	— 13 ± 4.6	8.9 ± 0.31
11 (23)	Sesame oil alternate days diet 1	273 ± 1.8	22 ± 3.5	10.1 ± 0.24

[1] Data are shown as means ± the standard error of the means.

[2] Daily food intake restricted to 6.0 gm, the mean daily food consumption of group 6.

[3] Daily food intake restricted to 9.0 gm, the mean daily food consumption of group 10.

groups were fed the diets for 21 days under the conditions and with the treatments indicated in table 3. Each animal was housed in an individual cage and daily determinations of food intake were made. The animals in groups 7 and 9 which were, respectively, the controls for groups 6 and 10, were restricted in food intake as noted in the footnotes to table 3. The animals in groups 9, 10 and 11 received subcutaneous injections of 0.1 ml sesame oil or 2.5 μg estradiol dipropionate in 0.1 ml sesame oil as indicated in table 3. All animals were given 3–4 drops of an oil concentrate of vitamin A and calciferol by dropper twice weekly to supply vitamins A and D. In order to prevent the animals chewing the paralyzed extremity, it was necessary to cut short the incisor teeth twice weekly. The animals were sacrificed and the humeri, after prolonged boiling of the fore-limbs in water, were dissected free of soft tissue. The bones were dried and rendered fat-free by continuous extraction in a Soxhlet apparatus for 48 hours with an equal part mixture of alcohol and ether. After the bones were dried and weighed, they were ashed to constant weight in platinum dishes in a muffle furnace at 700°C.

RESULTS AND DISCUSSION

The dietary regimens and other experimental conditions employed, the mean initial body weights of the animals, their mean post-operative gain in weight and their mean daily food consumption are shown in table 3. Table 4 is a presentation of the mean results obtained with the humeri. The percentage ash contents of the bones are expressed in relation to the dry, fat-free weights. The results pertaining to the total dry, fat-free and total ash weights of the humeri are expressed per 100 gm of initial body weight of the individual animals. These results are reported in this manner in order to permit a comparison, between the groups, of the mass of bone and of bone mineral in the humeri. This device is employed on the valid assumption that the initial dry, fat-free weights and ash weights of the bones were directly related to the initial body weights. The degrees of atrophy shown in the last column of

TABLE 4

Effects of dietary variations and of estradiol dipropionate on humeri of normal and paralyzed limbs of rats.[1]

GROUP	HUMERI OF NORMAL LIMBS			HUMERI OF PARALYZED LIMBS			
	Ash	Dry, fat-free wt. per 100 gm body weight	Ash wt. per 100 gm body weight	Ash	Dry, fat-free wt. per 100 gm body weight	Ash wt. per 100 gm body weight	Degree of atrophy
	%	*mg*	*mg*	%	*mg*	*mg*	%
1	67.47 ± 0.107	83.88 ± 0.763	56.71 ± 0.490	66.03 ± 0.142	72.60 ± 0.864	47.93 ± 0.605	13.7
2	65.97 ± 0.0833	76.08 ± 0.568	50.12 ± 0.374	62.49 ± 0.181	56.11 ± 0.474	35.08 ± 0.320	36.8
3	65.83 ± 0.123	78.31 ± 0.608	51.57 ± 0.401	63.42 ± 0.208	60.80 ± 0.835	38.20 ± 0.467	31.2
4	68.06 ± 0.134	82.68 ± 0.757	56.25 ± 0.522	67.26 ± 0.112	72.53 ± 0.828	48.78 ± 0.528	12.1
5	67.40 ± 0.154	82.10 ± 0.752	55.33 ± 0.418	66.35 ± 0.131	71.60 ± 0.699	47.51 ± 0.702	14.4
6	68.36 ± 0.132	68.96 ± 0.562	47.08 ± 0.359	67.03 ± 0.190	58.31 ± 0.721	39.09 ± 0.511	29.6
7	67.43 ± 0.0836	75.20 ± 0.668	50.71 ± 0.470	66.42 ± 0.122	65.66 ± 0.629	43.61 ± 0.480	21.4
8	68.14 ± 0.095	81.58 ± 0.911	55.58 ± 0.805	68.16 ± 0.140	81.46 ± 0.964	55.51 ± 0.581	...
9	68.08 ± 0.100	73.65 ± 0.720	50.14 ± 0.496	66.63 ± 0.126	62.47 ± 0.729	41.62 ± 0.504	25.0
10	68.79 ± 0.135	81.83 ± 0.718	56.07 ± 0.639	67.57 ± 0.189	73.55 ± 0.824	49.70 ± 0.622	10.4
11	67.91 ± 0.0872	80.00 ± 0.731	54.31 ± 0.466	66.90 ± 0.109	69.18 ± 0.728	46.28 ± 0.479	16.6

[1] The data are shown as means ± the standard error of the means.

table 4 were calculated from the differences between the mean total ash weight of the humeri of the paralyzed limbs and the average of the mean ash weights of the humeri of group 8. Table 5 is a compilation of significance ratios [2] derived from the mean results in table 4 and is presented to aid in the interpretation of differences between mean results in table 4.

[2] A difference of means is usually considered statistically significant if the significance ratio is 2.0 or greater than 2.0.

There is an indication of an increased rate of new bone formation (hypertrophy) in the humeri of the normal limbs of animals with unilateral brachial paralysis, as is shown by a comparison of the results obtained with the normal humeri of group 1 with the results obtained with the corresponding

TABLE 5

Significance ratios.[1]

	% ASH		DRY, FAT-FREE WT. PER 100 GM BODY WEIGHT		ASH WEIGHT PER 100 GM BODY WEIGHT	
	Normal	Paralyzed	Normal	Paralyzed	Normal	Paralyzed
			Group 1 compared with			
Group 2	11.1	15.4	8.2	16.7	10.6	18.8
Group 3	10.1	10.4	5.7	9.8	8.1	12.7
Group 4	3.4	6.8	1.1	0.06	0.6	1.0
Group 5	0.4	1.6	1.7	0.9	2.1	0.4
Group 6	5.2	4.2	15.7	12.7	15.8	11.2
Group 7	0.3	2.1	8.5	6.5	8.8	5.6
Group 8	4.7	..	1.9	..	1.2	..
Group 9	4.2	3.2	9.8	9.0	9.4	8.0
Group 10	7.7	6.5	2.0	0.8	0.8	2.0
Group 11	3.2	4.9	3.7	3.0	3.5	2.1
			Group 6 compared with			
Group 7	6.0	2.7	7.1	7.7	6.1	6.4
			Group 9 compared with			
Group 7	5.0	1.2	1.6	3.3	0.8	2.8
Group 10	4.2	4.1	8.0	10.1	7.3	10.1
			Group 10 compared with			
Group 11	5.5	3.1	1.8	4.0	2.2	4.4

[1] Difference of mean results divided by the square root of the sum of the squares of the standard errors of the means.

humeri of group 8. If this effect is real, the indication is that bone of a lower-than-normal degree of calcification was deposited in the normal humeri of group 1. This last suggestion is derived from the fact that although the dry, fat-free and total ash weights of the normal humeri of group 1 exceeded those of group 8 the percentage of ash in the normal humeri

of group 1 was significantly lower than that of the humeri of group 8. This evidence for hypertrophy of the humeri of the normal limbs is not in agreement with that of Riley, McCleary and Johnson ('45) who, however, used younger rats than those employed in this study.

The feeding of diets which were reduced to the lowest practicable content of calcium or phosphorus but which were otherwise adequate, obviously resulted in a reduction of the percentage of ash, the dry, fat-free and the total ash weights of the humeri of the normal limbs (group 1 compared with groups 2 and 3). It is also obvious that the atrophic process which occurred in the humeri of the paralyzed limbs was accentuated by diets greatly deficient in calcium or in phosphorus. The effects produced on the humeri of both the normal and paralyzed limbs by calcium deprivation appear to be more severe than those caused by a lack of phosphorus.

The effect of a calcium-enriched diet, containing *circa* 0.8% calcium (group 4) instead of *circa* 0.55% calcium present in the diet fed to the animals of group 1, was to increase the percentage of ash of the humeri of both limbs. However, the dry, fat-free and the ash weights of neither humeri were significantly affected by the diet of higher calcium content. These results indicate a limited ability of mature rats, in contrast to younger animals, to store extra calcium when an adequate diet is enriched with this element. There was no certain effect on either of the humeri resulting from a phosphorus-enriched diet (group 5) containing 0.51% phosphorus, instead of the 0.42% present in the diet fed to the animals of group 1.

The effect of a diet severely deficient in protein (nitrogen content 0.08% instead of 2.2%) was to increase slightly, but to a statistically significant degree, the percentage of ash in the humeri of the normal and paralyzed limbs (group 6). The total quantity of bone in the humeri of both limbs was, however, markedly reduced by protein starvation as is seen by comparing the results for dry, fat-free and ash weights with those obtained with group 1.

Group 7 was employed because of the fact that the animals of group 6 spontaneously limited their daily food intake to an average of 5.9 gm per day. On this account, part of the effects noted in the humeri of group 6 could have been due to a reduced intake of minerals and of calories by the animals. The diet fed to the animals of group 7 was the same as that fed to the animals of group 1 but was restricted to 6.0 gm per day per animal.

The results obtained from group 7 when compared with those of group 1 show that the percentage of ash of the humeri of the normal limbs was not affected, and that the effect on the percentage of ash of the humeri of the paralyzed limbs was at the borderline of statistical significance. However, the dry, fat-free and the ash weights of both humeri were reduced by partial starvation. This result could have been effected by an increased rate of bone resorption, thus producing osteoporotic bones. The results obtained with group 6 compared with those of group 7 show that the effects of protein deficiency on the skeleton are more severe than those which are produced by partial starvation equivalent to that experienced by the animals fed the protein deficient diet.

The animals of group 9 whose food intakes were less than their requirements but not as severely restricted as those of the members of group 7, nevertheless suffered more severely deleterious effects on their humeri than those experienced by the animals in the latter group. While no explanation can be offered for the mechanism of the anomalous results, they appear to be related to those for the sesame oil injections received by the members of group 9. Similar deleterious effects on the bones of normal and paralyzed limbs were found in group 11 which also received sesame oil injections but whose food intake was not restricted. These effects of sesame oil on the skeleton are additional evidence that this substance is not metabolically inert. Bruce and Tobin ('40) showed that daily intraperitoneal injections of 0.25 to 1.0 ml sesame oil interfered with weight gain of male rats whose initial body weights were about one-half those of the animals used in this study.

Evidence was also presented to show that injections of the alcohol-insoluble fraction of sesame oil reduced the rate of growth of the tibial diaphysis of suckling rats, and that the treatment prolonged the life of adrenalectomized immature rats and that of adrenalectomized lactating animals. Tobin ('41) found that sesame oil injections prolonged the life of adrenalectomized pregnant rats.

Group 9 is the proper control group for the animals receiving estradiol dipropionate (group 10). A comparison of the results obtained with these 2 groups shows that the effect of the administration of estradiol dipropionate was to reduce greatly the bony atrophy occurring in both humeri of the animals in group 9. This effect of estradiol dipropionate on bone metabolism is also seen when the results obtained with group 10 are compared with those of group 11; this effect is especially marked in the case of the humeri of the paralyzed limbs. It is probable that more markedly beneficial effects on skeletal disuse atrophy would have been obtained from the administration of estradiol dipropionate had the substance not been dissolved in sesame oil and had it not interfered with food consumption. These effects of estradiol dipropionate on bone furnish a direct verification of the effect of estrogenic hormones on the skeleton as deduced by Reifenstein and Albright ('47) from metabolic studies with cases of osteoporosis in the human.

SUMMARY

The effects of certain dietary alterations and of injections of sesame oil and estradiol dipropionate on the composition of the humeri of normal and denervated limbs of mature rats have been determined.

Severe calcium or phosphorus deficiencies caused a reduction in percentage of ash and in amount of bone in the humeri of the normal limbs. Both types of mineral deficiencies accentuated the atrophic process occurring in the humeri of the paralyzed limbs. These deleterious effects on bone were more marked in the case of calcium deprivation than in lack of phosphorus.

An increase of the calcium content of an adequate diet by 43% resulted in an increase of the ash content of the humeri of both limbs but produced no other significant effects on the bones. An increase of the phosphorus content of an adequate diet by 23% had no demonstrable effects on the bones of either limb.

Severe restriction of the daily intake of an adequate diet resulted in an atrophy of the humeri of the normal limbs and in an increase of the disuse atrophy of the paralyzed limbs. These deleterious effects were increased in animals receiving the same daily intake of minerals in a diet with a very low protein content.

Evidence was obtained indicating that the injections of sesame oil had a deleterious effect on the humeri of normal limbs and accentuated the atrophy of the humeri of the paralyzed limbs.

The administration of estradiol dipropionate in a dosage of 2.5 μg on alternate days was found to decrease the atrophy of the humeri of the normal limbs caused by the combination of sesame oil injections and food restriction, and to decrease the atrophy of the humeri of the paralyzed limbs.

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EFFECT OF PROTEIN INTAKE ON THE BONES OF MATURE RATS [1]

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The finding (Armstrong, '48) that severe restriction of the protein content of the diet of mature rats produced marked effects on the humeri suggested the present study of the influence of different levels of protein intake on the bones of such animals. While there is considerable information (Conner and Sherman, '36; Osborne and Mendel, '26; Quinn et al., '29) dealing with several phases of the influence of protein intake on skeletal growth of young rats, there appears to be little published knowledge with regard to the effects produced by protein deficient diets on the bones of mature animals.

METHODS

Diets containing respectively 4.5, 13.5, 9.0 and 18.0% lactalbumin as the source of protein [2] with the composition given in table 1 were prepared according to methods previously described (Armstrong, '48). Additional sucrose was employed, on an equal weight basis, as a replacement for protein in the 3 diets of lower protein content. The difference in the calcu-

[1] Supported by a grant from the Graduate School of the University of Minnesota. This paper was constructed from a thesis submitted by Miss Estremera to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the Master of Science degree.

[2] In addition to the small amount of protein contributed by the liver extract.

lated caloric contents [3] of diets 1 and 4, the 2 diets with the largest discrepancy in energy content, is 0.5%.

Male rats of the Sprague-Dawley strain, with weights of 240–260 gm when received, were fed commercial dog biscuit until their weights were within the range 260–265 gm when they were started on the dietary regimens. The 4 diets were fed to correspondingly numbered groups of animals, each consisting of 23 rats, for a period of 30 days. All animals were

TABLE 1

Composition of diets per kilogram.

DIET	1	2	3	4
	gm	*gm*	*gm*	*gm*
Lactalbumin — Borden's 15–42	180	135	90	45
Sucrose	400	445	490	535
Sucrose + vitamins [1]	200	200	200	200
Fat — (Spry)	169	169	169	169
Wesson's salt mixture	40	40	40	40
Liver extract (Wilson's fraction D)	10	10	10	10
Choline hydrochloride	1	1	1	1
Calories (calculated)	4,674	4,666	4,659	4,652

[1] Each 1000 gm of diet contained 2 mg thiamine hydrochloride, 2 mg pyridoxine hydrochloride, 3 mg riboflavin, and 4 mg calcium pantothenate.

either 99 or 113 days of age when the feeding of the synthetic diets was begun and individuals of the 2 age groups were distributed equally among the 4 groups. The principle of pair-feeding was employed in quadruplicate with sub-groups of 4 rats, each animal receiving a diet of different protein content, in order that the mean daily food consumption by the members of the 4 main groups should be equal. The "pacemaker" was almost always the animal receiving the diet of

[3] Calculated from the following caloric equivalent per gram: sucrose 3.94 Cal., fat 9.3 Cal., and lactalbumin 4.1 Cal. It seems possible from information relative to the composition of the lactalbumin used that the assumption that the caloric content of this material is 4.1 Cal. per gram errs on the high side. If this is a fact, it would have the effect of making more nearly equal the caloric contents of the diets.

lowest protein content. After the animals were sacrificed the dry, fat-free and ash weights of the humeri were determined by methods previously described (Armstrong, '48). The ash of the humeri of 4 animals each from groups 1 and 4 was dissolved in hydrochloric acid and analyzed for calcium (Clark and Collip, '25), and for phosphorus (Fiske and Subbarow, '25). The livers were weighed and sections of the organs from representative animals prepared for histologic examination. It was later realized that the animals, when started on the experimental regimens, were nearly, but not quite, at the end of the age period of measurable growth in length of humeri (Armstrong, '46). It thus became of interest to determine whether there were differences, among the groups, in length of these bones. Fortunately, the ashed bones, most of which had been retained, were fused and could be handled. Their length was determined to the nearest 0.1 mm with a vernier micrometer.

TABLE 2

Observations pertaining to the animals and results with regard to the humeri.[1]

	PROTEIN CONTENT OF DIET			
	18.0% (Group 1)	13.5% (Group 2)	9.0% (Group 3)	4.5% (Group 4)
Initial body wt. (gm)	262 ± 0.85	262 ± 0.78	262 ± 0.83	262 ± 0.47
Final body wt. (gm)	316 ± 3.4	302 ± 3.4	286 ± 4.4	248 ± 2.8
Daily food intake (gm)	12.1 ± 0.18	12.0 ± 0.17	12.0 ± 0.19	11.9 ± 0.23
Dry, fat-free wt. of humeri (gm)	0.2274 ± 0.00273	0.2251 ± 0.00260	0.2187 ± 0.00232	0.2093 ± 0.00264
Ash wt. of humeri (gm)	0.1537 ± 0.00148	0.1523 ± 0.00165	0.1483 ± 0.00134	0.1405 ± 0.00165
Calculated ash content (%)	67.65 ± 0.307	67.66 ± 0.26	67.82 ± 0.34	67.21 ± 0.36
Length of humeri (mm)	26.27 ± 0.0784	26.33 ± 0.15	26.00 ± 0.106	25.65 ± 0.160

[1] The data are shown as means ± the standard error of the means.

RESULTS AND DISCUSSION

Table 2 shows the results. All data referring to the humeri are the means of the average results obtained with the 2 bones from individual animals in each group.[4] The data pertaining to the humeri are not recorded in relation to the initial body weight, as in a previous report (Armstrong, '48), because the mean initial body weights of the animals in the 4 groups were identical with small variations from the means.

TABLE 3

Difference between mean results of experimental groups and those of control group and tests for probable significance of difference of the means.

GROUP	DRY, FAT-FREE WT. OF HUMERI		ASH WT. OF HUMERI		LENGTH OF HUMERI	
	Diff.[1]	S. R.[2]	Diff.[1]	S. R.[2]	Diff.[1]	S. R.[2,3]
	%		%		%	
2	— 1.01	0.61	— 0.92	0.63	+ 0.23	0.35
3	— 3.83	2.4	— 3.51	2.7	— 1.03	2.0
4	— 7.96	4.77	— 8.59	5.96	— 2.36	3.48

[1] Difference of mean results expressed as a percentage of the mean result obtained with the control group (group 1).

[2] S. R. = Significance ratio.

[3] Substitution of S. R. with Fisher's "T" does not affect the interpretation.

Table 3 is a presentation of the differences between the results obtained with group 1 (control) and with each of the 3 experimental groups expressed as percentages. This table also includes the values of the significance ratios [5] to aid in the interpretation of significance of differences between the mean results of the control and experimental groups.

It will be noted that the weight gain of the animals varied in the same order as the protein content of the food, even though all animals received essentially equal caloric intakes during the experimental period. An interesting result which

[4] Group 1, length of humeri — 18 animals; group 4, length of humeri — 17 animals. In all other cases, 23 animals are represented in the means.

[5] Difference of means divided by the square root of the sum of the squares of the standard errors. A difference of means is usually considered statistically significant if the significance ratio is 2 or greater than 2.

emphasizes this point is the fact that the animals of group 1 gained 54 gm, on an average, while those receiving a diet containing 4.5% protein lost, on an average, 14 gm. The difference in energy intake over the 30-day period between groups 1 and 4, taking into consideration their food consumption and the calculated caloric equivalents of the 2 diets, is 36 Calories, which could account for only 4–5 gm of weight difference (as fatty tissue) between the animals of these 2 groups. Forbes and coworkers ('35, '40) have also shown, with growing and with mature rats, that the weight gain and energy disposition of animals receiving equal total caloric intakes are dependent on the protein content of the diet. These workers, however, did not employ diets containing less than 10% protein. They observed a diminished loss of energy in the feces by the animals receiving the higher amounts of protein, but the metabolized energy decreased in the reverse order. A diminished total heat production by the animals receiving the higher amounts of protein was observed which, by elimination, was attributed to a lowered spontaneous activity since neither the basal heat production of the animals of the several groups nor the estimates of the specific dynamic action of the various diets were different (Forbes et al., '35). More recently Forbes and Swift ('44) have presented evidence that the specific dynamic action (S.D.A.) of foodstuffs fed as a mixture is not the sum of the specific effects of the components. While it is not possible to state whether the S.D.A. of the food fed group 1 of this study was significantly less than that fed the other groups, this possibility is suggested by the work of Forbes and Swift ('44), and this point would bear further investigation. It would also be of interest to determine whether the animals receiving the diets of lower protein content actually expended more energy in activity than the animals receiving the higher amounts of protein. The observations of Slonaker ('31) in regard to the effect of protein intake on spontaneous activity are not entirely pertinent to the present study since he employed diets of 10, 14, 18, 22 and 26% protein content fed *ad libitum* to rats. He did not observe any regular order of ac-

tivity of his animals, as determined in revolving cages, to be correlated with the protein content of the diet.

The results pertaining to the humeri show that the bones of the animals consuming the diet containing 13.5% protein (group 2) had only slight and statistically insignificant lower dry, fat-free and total ash weights than those of the control group. The humeri of the animals of groups 3 and 4 clearly suffered a significant reduction in total dry, fat-free and ash weights. It appears, from the results obtained with group 3, that 9.0% protein in the diet is near the level of dietary protein required to maintain the skeleton of rats, such as those used in this study. There appears (table 3) to have been, in group 4, a slightly greater reduction of ash weight than of total dry, fat-free weight of the bones. Thus, the bones of the animals fed a diet of 4.5% protein lost (or failed to gain in the process of bone reformation) slightly more mineral than they did of organic material. The mean result for percentage ash of the humeri obtained with group 4 is in the direction that would be expected to result from a greater reduction in total ash weight than in dry, fat-free weight, but does not differ statistically from the mean result obtained with the control group.

It has been shown (Armstrong, '46) that the humeri of rats of the strain used in this study continue to grow in length, but at a reduced rate, until an age is reached which is approximately equal to the sacrifice age of the animals used in this study. Thus, the possibility existed that the lower dry, fat-free and ash weights of the humeri of the animals in groups 3 and 4 were due to an interference in bone formation, in the sense of bone growth. The data given in table 3 show that an interference of bone growth is a factor which accounts for a part, but not all, of the differences of the humeri of groups 3 and 4 from those of group 1. While the humeri of groups 3 and 4 were significantly shorter than those of group 1, the lesser lengths of these bones were not in proportion to their lower dry, fat-free and total ash weights. This deduction is made from a comparison of the percentage deviations of the mean results of groups 3 and 4 with those of group 1.

The calcium and phosphorus contents of the ash of the humeri of groups 1 and 4 were not found to be significantly different. The weights of the livers in the 4 groups were closely correlated with their final body weights (averaging 4.79 gm per 100 gm body weight) and sections of this organ from typical animals were normal in histologic structure.

SUMMARY AND CONCLUSIONS

The effects on body weight and on the humeri of rats produced by feeding to mature rats for 30 days equal amounts of essentially isocaloric diets containing 4.5, 9.0, 13.5 and 18.0% protein as lactalbumin have been determined. The following conclusions are drawn:

1. A diet containing 4.5% protein will not support body weight of mature rats while diets containing 9.0, 13.5 and 18.0% protein permit an increase in body weight in approximate proportion to the protein content.
2. A diet containing 13.5% protein supports a degree of skeletal constitution in mature rats equal to that of a diet containing 18.0% protein.
3. Diets containing 9.0 and 4.5% protein are inadequate to support normal skeletal constitution in mature rats.
4. Diets containing 9.0 and 4.5% protein interfere with the growth in length of the humeri of rats which are near the end of the period of skeletal growth, but retardation of skeletal growth does not account for all the skeletal changes caused by feeding protein deficient diets.
5. The composition of the bone ash is not affected by feeding protein deficient diets to mature rats.

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URINARY EXCRETION OF ASCORBIC ACID BY GUINEA PIGS AT DIFFERENT AGES

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TWO FIGURES

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Few studies have been made of the urinary excretion of ascorbic acid in guinea pigs and none have dealt with the question of age. Hess and Benjamin ('34) reported that guinea pigs on an oat diet devoid of ascorbic acid excreted only 0.146 mg each per day, those with a normal intake (carrots and lettuce) excreted 0.3 mg and those with a very high intake (10–60 ml orange juice) excreted only 0.167 mg. No difference was observed between normal animals and those which had moderate scurvy, either as to the initial indophenol-reducing values of the urine or in the values showing the urinary response to ingestion of the vitamin. However, when ascorbic acid was injected intraperitoneally in large amounts similar to the quantities which had been administered orally a sharp rise in urinary excretion occurred, usually on the second or third day after the injections were started. When the injections were stopped, the reduction in urinary concentration of the substance was often quite gradual. Von Euler and Memberg ('35) also failed to find an appreciable excretion of ascorbic acid in the urine of guinea pigs when the vitamin was administered orally.

It is possible that the low values reported by these investigators for urinary excretion of ascorbic acid in guinea pigs on a scorbutogenic diet, or even in normal animals receiving the vitamin orally, may have been due not to the presence of traces of ascorbic acid but to other indophenol-reducing substances.

Zilva ('36) studied the ascorbic acid requirement of the guinea pig and found that an oral dose of 500 mg is necessary for growing guinea pigs (300 gm) before the vitamin is excreted. Von Euler and Memberg ('35), however, had found little excretion with doses as high as 1 gm. In agreement with Hess and Benjamin ('34), Zilva found that urinary excretion is brought about by much smaller doses when the vitamin is injected than when it is administered orally. Ascorbic acid appeared first in the urine when 20 mg was injected. This was the smallest amount which produced the maximum concentration in the tissues. As the dose was increased, the amount excreted rose also.

The problem of the retention of ascorbic acid by humans of different ages has not been given extensive study. In much of the work that has been done thus far, data have been obtained which afford interesting and valuable suggestions but the studies have been concerned with too few subjects and over periods too short to furnish conclusive evidence of an effect of age. An investigation bearing on this problem, which should be specially noted, was conducted by Schuck and Purinton ('43). They followed the daily excretion of ascorbic acid in a group of 63 subjects — 52 women and 11 men. They used the saturation test with 500 mg of ascorbic acid administered intravenously. The individual to be tested was given a definite diet, with an ascorbic acid intake of 15 mg daily for the males and 14 mg for the females. After determining the quantity of the vitamin excreted during the first 24 hours after the test dose, Schuck and Purinton calculated the quantity retained by the individual. By subtracting the quantity of ascorbic acid excreted during the test period from the total amount administered, a retention figure was obtained.

Then, by subtracting the value for the ascorbic acid excreted under fasting conditions from the retention figure, a value was obtained which was considered as the quantity of ascorbic acid metabolized and probably therefore required by the individual in question. By this method a metabolism of 113 mg was obtained for the women subjects under 20 years of age, whereas for women between 25 and 50 years of age only 81 mg was metabolized. It is possible that still greater differences might have been found had there been wider differences in age between the various groups, particularly if some were in an active stage of growth. Women between 20 and 25 years of age metabolized slightly more ascorbic acid per unit of body weight than men in the same age group. A somewhat different interpretation of the metabolism data could be made if it were assumed that the subjects may not have been saturated at the beginning of the tests, notwithstanding the fact that they were excreting a small amount of the vitamin during the fasting period. If the younger group, for example, were in a higher degree of unsaturation than the others, the amount metabolized would consist of the average daily amount plus the retention of an additional amount because of the unsaturation. In other words, the vitamin would be used up not only for the day's needs but to make up for the previous deficit as well.

No reports have been made of estimations of ascorbic acid retained by the same individuals at different ages either in man or in animals. Measurements of this kind on humans have not been made up to the present time because of the length of time required and also because of the difficulty of maintaining satisfactory dietary conditions for such extended periods. Data obtained in such investigations would be of considerable interest because of their possible bearing on the ascorbic acid requirement of the individual at different ages. Since guinea pigs pass through the various phases of their life cycle rapidly as compared to humans and since dietary control is a simple matter, they were selected as the experimental subjects for tests of this kind.

PROCEDURE

Guinea pigs of an inbred strain[1] were placed in metabolism cages at ages ranging from 15 to 26 days and were fed a pelleted scorbutogenic stock diet (commercial) supplemented with 1% of powdered yeast. Fresh food and water were supplied daily. The animals (8 males and 3 females) were weighed daily and were given ascorbic acid intraperitoneally in the proportion of 5 mg per 100 gm of body weight. The solution for injection was prepared by dissolving 15 mg of the vitamin per milliliter of a 0.5% solution of $NaHCO_3$. The animals were kept in 8-inch metabolism cages until 3 months of age, when they were transferred to 10-inch cages. The animal room was maintained at 76 to 80°F.

Five grams of crystalline metaphosphoric acid were placed daily in each bottle for collecting the urine. During the early phases of the experiment this amount appeared to be sufficient for protecting the ascorbic acid against oxidation. Later, as the volume of urine increased, the amount of metaphosphoric acid was also increased. By the time the animals were approximately 4 months old the amount had been raised to 10 gm and this quantity was used for most of the animals for the remainder of the test. For 2 animals which produced an unusually large volume of urine, 12 gm of the acid were used. Control tests were also conducted to determine the ability of the metaphosphoric acid to protect solutions of pure ascorbic acid during a 24-hour period. The amounts of the acid used were found to be adequate under the experimental conditions. Occasional tests were also made with urine added to the ascorbic acid solution.

Determinations of the amount of ascorbic acid excreted in the urine were made daily by the indophenol titration method and approximately every 10 days by the osazone method of Roe and Kuether ('43). The tests were continued by both methods

[1] Animals from family no. 13 were used. This strain was developed by the Genetics Section of the Bureau of Animal Industry of the United States Department of Agriculture. Later, the line was continued by the Genetics Section of the National Cancer Institute, from which the animals here used were obtained.

until the animals reached an age of 6 months and then for 2 months longer by the indophenol method only.

RESULTS

Per unit of body weight, both methods of assay showed a definite upward trend in excretion with increasing age, leveling off at about the time growth was nearing completion. Figure 1 shows the curves for male animals at successive ages, the

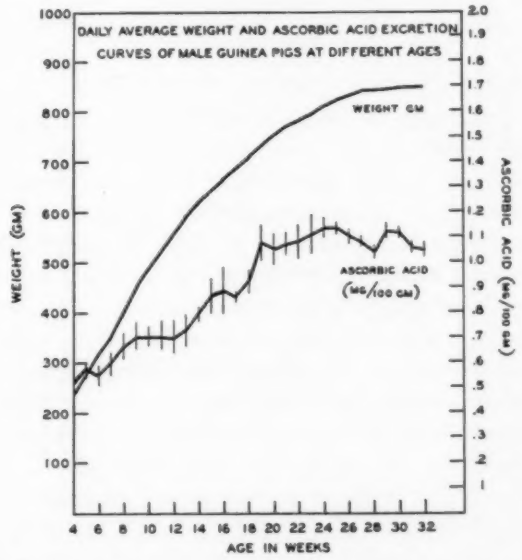

Fig. 1 Daily average weight and ascorbic acid excretion curves of 8 male guinea pigs at different ages.

average values for weight being expressed in grams, and those for excretion of ascorbic acid as milligrams per 100 gm of body weight. The standard deviations for the excretion values obtained with the indophenol method are also shown. Because of the infrequency of the determinations by the osazone method, the excretion values yielded by this technique have not been plotted. In general, there was fair agreement

in the results obtained by the 2 methods. There were not enough female animals to permit construction of growth and excretion curves comparable to those shown for the male animals. The excretion values of the females tended to be more fluctuating than those of the males, and after sexual maturity they were definitely lower than those of the male animals. Of the total ascorbic acid administered, 10.6% was excreted

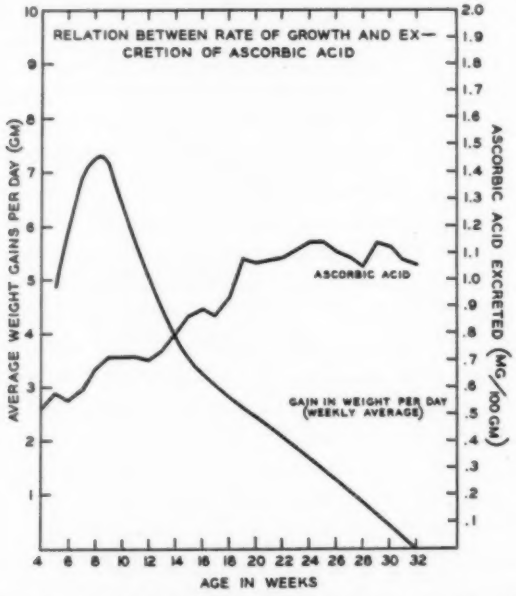
RELATION BETWEEN RATE OF GROWTH AND EX— CRETION OF ASCORBIC ACID
AVERAGE WEIGHT GAINS PER DAY (GM)
ASCORBIC ACID EXCRETED (MG/100 GM)
ASCORBIC ACID
GAIN IN WEIGHT PER DAY (WEEKLY AVERAGE)
AGE IN WEEKS
4 6 8 10 12 14 16 18 20 22 24 26 28 30 32

Fig. 2 Relation between rate of growth and excretion of ascorbic acid.

in the urine at the age of 6 weeks, 13% at 9 weeks, and 23% at 24 weeks of age. Figure 2 shows the relation between the rate of growth of male guinea pigs and the excretion of ascorbic acid per unit of weight. The period of most rapid gain in weight in both sexes, that is, up to the twelfth or thirteenth week (the maximum gains occurring during the eighth and ninth weeks) is the time of lowest rate of excretion of the vitamin per unit of body weight. There is an inverse relation

between the general direction of the 2 curves, the excretion per unit of body weight being, in general, lowest when the weight-rate gains are highest. As the rate of growth diminishes, the excretion rises.[2] The increase in excretion per unit of body weight continues for the male animals until the twenty-fourth week and for the females until around the twenty-first week. From the fourteenth to the nineteenth week the rate of growth falls off only moderately whereas ascorbic acid excretion per unit of weight rises markedly.

DISCUSSION

There was considerable variation throughout the entire experimental period in the excretion of ascorbic acid from day to day. If the values were high 1 day, they tended to be lower the next. The weekly averages thus give a better indication of the excretion than the values for single days.

Both the excretion and the growth for the period extending from the third to the ninth week require further study. In another test it would be desirable to begin the injections shortly after birth and also to use a higher dosage so that when the excretion and growth studies are begun at the time of weaning the animals would be saturated, or nearly so. It is probable, however, that at this age a single injection a day even with a relatively high dosage would be insufficient to maintain a saturated condition throughout a 24-hour period. It is also possible that with the animals saturated, or nearly so, the excretion curve would be practically flat from weaning time to the fourteenth week instead of showing the rise here found from the fifth to the ninth or tenth week. It is also possible that, with conditions permitting the maintenance of a favorably high blood level of ascorbic acid throughout each 24-hour period, the growth curve would show a plateau at a high

[2] In other tests it has been found that with respect to the retention of ascorbic acid (Reid, '47a), mature animals which are required to build new tissues in the healing of wounds behave apparently like younger animals during the healing period.

level during this early period instead of a rise in rate to a peak at the seventh week.

The present results have shown a difference in excretion of approximately 0.4 to 0.5 mg/100 gm between the 8 week-old and the adult animal 6 months old. Presumably this may represent an amount of the vitamin actually used up daily by 100 gm of tissue during the growing period. For a 300-gm animal injected intraperitoneally, there would thus be a daily utilization of 1.2 to 1.5 mg. This is approximately the daily requirement of the vitamin as reported by Dann and Cowgill ('35). They found that with oral administration 1 ml of lemon juice (0.4–0.6 mg ascorbic acid) per 100 gm of body weight and irrespective of age is required to protect guinea pigs from scurvy, as measured by the Höjer tooth method.

Results of the present tests suggest strongly, though they do not prove, that the ascorbic acid requirement of the young animal is greater than that of the adult. The possible discrepancy between these results and those of Dann and Cowgill may be caused by the difference in mode of administration of the vitamin.

Results obtained in later studies (Reid, '47b) suggest that the major portion of the intraperitoneally injected vitamin is destroyed in the contents of the digestive tract. It appears possible, however, that the 10% greater loss in the growing animal at 9 weeks as compared to that in the adult animal at 6 to 8 months may constitute a fraction disappearing in a different way. This 10% fraction may conceivably be used up in the body tissues in growth. The fact that the excess amount which disappears during growth is the same as the amount known to be required by the young animal lends some support to this hypothesis. However, the possibility of the loss occurring in other ways is recognized.

SUMMARY

A study has been made of the retention of ascorbic acid by guinea pigs at different stages of growth. The animals were fed a scorbutogenic diet supplemented daily with as-

corbic acid injected intraperitoneally in relation to body weight.

The results of daily determinations of the ascorbic acid excreted in the urine, per unit of body weight, show a definite upward trend with increasing age, leveling off at about the time growth is nearing completion. The excretion at 8 weeks of age was 0.67 mg/100 gm and at 24 weeks, 1.14 mg. Of the total ascorbic acid administered, 10.6% was excreted in the urine at the age of 6 weeks, 13% at 9 weeks, and 23% at 24 weeks.

An inverse relation was observed between the excretion of ascorbic acid per unit of body weight and the rate of growth at different ages.

The results show that a greater amount of ascorbic acid disappears during the period of rapid growth than during adulthood. Further study is necessary before conclusions can be drawn as to an effect of age on the ascorbic acid requirements of the guinea pig.

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